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A role for glutathione in reactions between oxidants and proteins

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A role for glutathione in reactions between oxidants and proteins.

by

Robert Joseph Mallis

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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Major: Biochemistry

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GENERAL INTRODUCTION

Cells are exposed to oxidative insult throughout the life span of an organism. Cellular respiration relies on single electron reductions of molecular oxygen to water in order to produce energy, so that intermediate reactive oxygen species are continually being generated by mitochondria. Some of these reactive intermediates inevitably escape into the cytoplasm, causing a steady flux of reactive oxygen species within cells. Immune cells generate hydrogen peroxide, superoxide, nitric oxide and peroxynitrite when activated as part of host defense in higher organisms. Cancer cells have been shown to produce hydrogen peroxide at levels equivalent to the oxidative burst of neutrophils (1). Several disease states including cancer (1,2), immune disorders (3), neurodegenerative disorders (4) and aging (5) are accompanied by oxidative phenomena. Within cells, proteins are susceptible to damage in these oxidative conditions (5). Cysteine, because of its reactive sulfhydryl-containing sidechain, is particularly susceptible to oxidative modification (6). Modification of cysteine residues of proteins has been shown to occur within cells under diverse oxidative conditions (7-11), and has been shown to inhibit the activity of purified proteins (12,13,14). Oxidation of protein cysteine residues thus has the potential of permanently inactivating proteins and disrupting their normal functions.

Glutathione function in cells

Organisms have evolved mechanisms for eliminating excess reactive oxygen species. This includes enzymatic systems such as superoxide dismutase, catalase, and glutathione

peroxidase. Low molecular weight antioxidants, such as ascorbate, vitamin E and glutathione (GSH)[1,2] act as scavengers alone or as a part of enzymatic systems. The tripeptide GSH is able to protect cells from oxidative insult, although the mechanism for this protection is not completely understood. It is proposed that GSH acts as a reductant of reactive oxygen species (ROS) either on its own (15-18) or in conjunction with glutathione peroxidase, reducing the oxidant and in the process being converted to glutathione disulfide (GSSG):

$$[1]\ GSH + ROS_{ox} \rightarrow GSSG + ROS_{red}$$

GSSG can be reduced back to GSH by glutathione reductase at the expense of cellular NADPH:

$$[2]\ GSSG + NADPH \xrightarrow{\text{Glutathione Reductase}} 2GSH + NADP^{+} + 2H^{+}$$

Protein S-glutathiolation

GSH may also act in concert with reactive cysteine residues on proteins to protect these same residues from oxidative damage (6,7,19):

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[1] Abbreviations used in this dissertation: AAPH, 2,2'-azobis(2-amidinopropane)dihydrochloride; DTT, dithiothreitol; Erk, extracellular signal-regulated kinase; GSH, reduced glutathione; GSSG, glutathione disulfide; IAA, iodoacetic acid; IAM, iodoacetamide; IEF, isoelectric focusing; MEK, Mitogen activated protein kinase/Erk kinase; NEM, N-ethylmaleimide; ROS, reactive oxygen species.

[2] A note on nomenclature: GSH refers ONLY to the reduced form of glutathione. GSSG refers only to glutathione disulfide. Oxidized glutathione may refer to several forms of glutathione including GSSG, cysteine-glutathione disulfide, S-glutathiolated protein and S-nitrosoglutathione.

[3] $GSH + ProteinSH + ROS_{ox} \rightarrow ProteinSSG + ROS_{red}$

Protein cysteines can be S-glutathiolated in cells by a variety of oxidants including activated neutrophils, monocytes, H_2O_2, superoxide, t-butyl hydroperoxide, menadione, and diamide (7-10,19). Several proteins that are readily S-glutathiolated in cells include carbonic anhydrase III (20), actin (8), hemoglobin (21), and glyceraldehyde-3-phosphate dehydrogenase (9). A role for protein thiols in protection of cells from oxidative damage has been proposed (6,21,22,23), but reaction rates of protein thiols with oxidants such as H_2O_2 or superoxide might not be sufficiently fast to compete with other antioxidant systems to make a significant contribution (24). In cells, protein S-glutathiolation is a transient event. However, the mechanism for the reduction of S-glutathiolated proteins is not well characterized. The protein-glutathione mixed disulfide, known as an S-glutathiolated protein, could be converted back to the reduced protein by another molecule of GSH (20,25):

[4] $ProteinSSG + GSH \rightarrow ProteinSH + GSSG$

Cellular dithiol proteins such as glutaredoxin or thioredoxin (25) could also reduce the S-glutathiolated protein:

[5] $ProteinSSG + Protein(SH)_2 \rightarrow ProteinSH + Protein(SSG)SH$

GSSG would then be reduced as in equation 2, while the dithiol protein could be reduced as in equation 4 or undergo internal thiol-disulfide exchange:

[6] $Pr(SSG)SH \rightarrow PrS_2 + GSH$

The oxidized disulfide protein product of equation 6 could be reduced by other dithiol proteins, or be reduced by a dithiol reductase like thioredoxin reductase. The appearance of S-glutathiolated proteins in cells under oxidative stress (7-10,19) and the identification of glutaredoxin as a specific dethiolase capable of reducing protein-glutathione mixed disulfides

(25), suggest that the interaction of GSH with proteins is a significant antioxidant function of GSH in cells.

Irreversible oxidation of protein cysteine

The cytosolic protein, carbonic anhydrase III, has been shown to be S-glutathiolated *in vivo* by oxidants including diamide, t-butyl hydroperoxide, menadione, H_2O_2, and superoxide (7,19,25). Additionally, purified carbonic anhydrase III is S-glutathiolated by H_2O_2, diamide, GSSG, and superoxide (7,19,20). In conditions of GSH depletion in cells, menadione has been shown to cause irreversible oxidation, i.e., oxidation of cysteine to a state that is not reducible by thiol-disulfide exchange, of carbonic anhydrase III (7). Xanthine/xanthine oxidase can cause irreversible oxidation of purified carbonic anhydrase III when GSH is not present to protect protein cysteines (19). Thus, in the absence of GSH, the reaction of protein with an oxidant does not proceed as in equation 3, but rather as in equation 7:

$$[7]\ \text{ProteinSH} + \text{ROS}_{ox} \rightarrow \text{ProteinS}_{ox} + \text{ROS}_{red}$$

This oxidized form of protein could not be reduced by equations 4-6 but instead would permanently lose the function of the cysteine residue. If this cysteine were part of the catalytic center of a protein, then the protein would be rendered inactive. These irreversible oxidation products have not been very well characterized. Based upon studies of oxidation of low molecular weight thiols, it is likely that these oxidation products are cysteine sulfinic or cysteine sulfonic acid (15). Reactions of purified monoalkylated glutathione reductase with H_2O_2 have been shown to produce cysteine sulfonic acid (13). Sulfinic acid formed in the active site of purified glutathione reductase that was incubated with dinitrosyl-

diglutathionyl-iron as determined by X-ray crystallography (26). Similarly, sulfinic and sulfonic acid have been found at the active site of NADH peroxidase (27). These experiments demonstrate the formation of irreversibly oxidized protein cysteine residues. Developing an assay for irreversibly oxidized cysteine may be a critical step in understanding the interactions between GSH and protein cysteines.

Oxidative regulation of signal transduction

Oxidation may play an important role in the degeneration and loss of control of signal transduction pathways that is associated with aging, cancer, and other disease states (1,2,5,28-30). It is well known that nitric oxide (NO) regulates vascular relaxation through initial stimulation of guanylate cyclase by NO (31). This produces cyclic guanosine monophosphate (cGMP), which interacts with signaling pathways to produce the vascular relaxation. However, NO plays multiple roles in cells, including direct oxidation of proteins and glutathione (32,33). For example, NO can prevent apoptosis through direct modification of the active site cysteine of caspase-3 (32). NO and other reactive oxygen species may also contribute to tyrosine phosphorylation of proteins in cell by inhibiting tyrosine phosphatases (14,34,35).

The role of reactive oxygen species in signal transduction may not be limited to inhibition of protein activity. For instance, stress-inducible transcription is activated by oxidative modification of OxyR, NF-κB, and AP-1 (36-38). Apoptosis appears to be inhibited by H_2O_2 soon after treatment of cells through direct inhibition of caspase-3 (39). However, if the initial dose of H_2O_2 is above a threshold level, apoptosis may be initiated at a later time (39). Thus, the role of H_2O_2 in apoptosis is complex and depends on more than

simple inhibition of caspase-3. 3-Morpholinosydnonimine (SIN-1), a compound that generates superoxide and nitric oxide appears to regulate the lipidation of proteins in neuronal cell lines (40). This suggests that reactive oxygen species may also regulate proteins through subcellular localization of proteins to cellular membranes.

Ras

Ras is a guanine nucleotide binding protein that is essential for proliferation and differentiation in many eukaryotic cell types (41). Ras is an intermediate in the well-characterized extracellular signal-regulated kinase (Erk) pathway, a signal transduction cascade which begins at a membrane bound receptor and ends in the nucleus (41,42). Binding GTP (43) activates Ras. This binding occurs very slowly because it requires the release of GDP, which inactivates Ras. However, when effectors upstream of Ras are activated, guanine nucleotide exchange factors (GEFs) stimulate Ras to release GDP and bind GTP (44,45). This activated Ras then will bind and activate Raf, which can phosphorylate and activate MAP kinase/ERK kinase (MEK). MEK phosphorylates and activates the Erks, which translocate into the nucleus and activate transcription of genes. When Ras hydrolyzes GTP to GDP (46), it becomes inactive once again. Because the endogenous GTPase activity of Ras is very low, GTPase activating proteins (GAPs) are necessary for inactivation of Ras by stimulating its GTPase activity (44,45).

Ras mutants that are unable to hydrolyze GTP, or are otherwise unable to become inactive, are oncogenic, causing uncontrolled proliferation of cells. Activated forms of Ras are found in >30% of all human cancers (44). While Ras is central to the Erk pathway,

interactions with many different signal transduction cascades seem to be a necessary part of the cellular function of Ras (41).

H-Ras

Ras proteins exhibit a high degree of homology over much of the protein, but vary significantly near the C-terminus. The C-terminus of H-Ras, one of 4 closely related Ras family members, contains three cysteine residues that impart unique biochemical properties that are not entirely understood on the biological level (47,48). One cysteine, Cys186, is farnesylated, while the other two C-terminal cysteines, Cys181 and Cys184, are palmitoylated *in vivo*. Farnesylation is the addition of a C_{15} isoprenoid to the cysteine via a thioether linkage. Farnesylation is catalyzed by a farnesyl transferase that specifically recognizes a four amino acid motif that occurs at the C-terminus of H-Ras (i.e. CaaX). Farnesylation is thought to localize H-Ras to the plasma membrane and may also play a role in further localization within that membrane (47). H-Ras loses its biological activity if farnesylation is prevented (49-52). Palmitoylation of Cys181 and Cys184 is a reversible modification via a thioester linkage. Membrane localization is possible without palmitoylation, but biological activity and the ability of oncogenic mutants of H-Ras to cause transformation is reversed when one or both of these cysteines is mutated (48). No enzymes that catalyze palmitoylation or depalmitoylation of H-Ras have been found to date. The biological function of palmitoylation is still uncertain.

Oxidative modulation of the Erk pathway

X-irradiation, H_2O_2, superoxide, NO, S-nitroso-N-acetylpenicillamine, and sodium nitroprusside have all been found to promote activation the Erk pathway in cell culture systems (52-55). The degree of activation and the kinetics of this activation appear to depend on the source and dose of the reactive oxygen species applied (53,54). Erk activity is maximally induced by an NO concentration of about 1 μM (53). At higher NO concentrations the Erks are not activated (53). This increase in activity at low NO concentrations and decrease in activity at higher concentrations suggests that there are multiple factors which determine Erk activation by reactive oxygen species. While 50 μM H_2O_2 was able to promote maximal activation of Erk2 in NIH-3T3 and smooth muscle cells, 200 μM H_2O_2 was necessary for maximal activation in HeLa, Rat1 and PC12 cells (54). This suggests some cell specificity for the activation of Erk by reactive oxygen species.

Several studies have shown a relationship between activation of the Erks and thiol oxidation. This was shown either by adding N-acetylcysteine to cell cultures or by depleting cellular GSH before oxidant stimulation (53,54). This suggested that direct oxidation of a protein might be responsible for activation of the Erk pathway. For instance, the epidermal growth factor (EGF) receptor may be directly activated by H_2O_2 through thiol oxidation, leading to activation of the Erk pathway (54). The tyrosine phosphatase that normally inactivates Erk may be inhibited by direct oxidation of the active site cysteine (54). Dominant negative Ras, Ras-N-17, prevented activation of Erk by H_2O_2, suggesting a role for Ras (54). Activation of Erk by NO donors in cells was also prevented by overexpression of a C118S mutant form of H-Ras and may therefore be a result of activation of H-Ras by S-nitrosylation of Cys118 on H-Ras (56). S-nitrosylated Cys118 on H-Ras was produced as a

result of incubation of a purified truncated mutant of H-Ras (Δ167-190) with NO (56). The GTP/GDP ratio of purified truncated H-Ras was found to be increased with S-nitrosylation of the protein (56), and H-Ras immunoprecipitated from Jurkat T cells treated with NO was also found to have an elevated GTP/GDP status (57). H-Ras seems to be a pivotal participant in Erk activation by reactive oxygen species, but inhibition of several other participants in the Erk signaling pathway individually or in combination also can prevent this activation.

Dissertation organization

This dissertation contains a general introduction, three chapters, each of which will be modified according to the preferences of individual journals and submitted for publication, and a general summary and conclusions section. References for the general introduction and general summary and conclusions sections appear at the end of the dissertation. All of the experiments described in the three chapters were performed solely by the author with the helpful advice and discussion of Dr. James A. Thomas, with the exception of 1C, 5B and 5C in Chapter II which were performed by Yanbin Ji.

CHAPTER I:

GLUTATHIONE-MEDIATED PROTECTION OF CARBONIC ANHYDRASE III BY S-GLUTATHIOLATION

A paper to be submitted to Archives of Biochemistry and Biophysics

Robert J. Mallis and James A. Thomas

Abstract

This report presents a study of the reaction of carbonic anhydrase III, a cytosolic protein which contains two reactive cysteine residues, with two oxidants, hydrogen peroxide (H_2O_2) and the peroxyradical generator 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH). The role of reduced glutathione (GSH) in these reactions was explored. Carbonic anhydrase III reacts with both oxidants, producing irreversibly oxidized cysteine residues in the absence of GSH. In the presence of low concentrations of GSH (approximately equimolar to protein thiol concentrations) irreversible oxidation of carbonic anhydrase III is prevented and S-glutathiolation is observed. Thus, S-glutathiolation blocks sulfhydryl oxidation, preventing irreversible damage. Equivalent concentrations of glutathione disulfide (GSSG) do not produce significant S-glutathiolation. These experiments suggest that a variety of oxidants including H_2O_2 and peroxyradicals cause S-glutathiolation by a mechanism that involves a direct oxidation of a protein sulfhydryl and subsequent reaction

with GSH to produce the S-glutathiolated protein. Glutathione disulfide (GSSG) is not an important intermediate in S-glutathiolation caused by either of the two oxidants studied. This suggests that S-glutathiolated proteins can form in cells in the absence of appreciable GSSG formation, and may provide a mechanism for reversible inhibition of protein sulfhydryl-containing proteins during oxidative stress. These experiments also show that in the absence of sufficient GSH, oxidation reactions lead to irreversible protein sulfhydryl damage.

Introduction

Glutathione (GSH)[1,2] is an essential component in the protection of cells from oxidants. It is thought to prevent oxidation of proteins by two major mechanisms. First, it may function as a direct scavenger of reactive oxygen species (1,2,3,4). This first function allows proteins to avoid becoming damaged during oxidative events. Second, it can form mixed disulfides with proteins, termed S-glutathiolation of proteins (5,6). This is a reversible oxidation of protein cysteine residues that may prevent further oxidation (6,7). Both the oxidation of GSH to glutathione disulfide (GSSG) and the S-glutathiolation of proteins occur within seconds of addition of oxidants to cell culture and are among the earliest measurable reactions to occur during an oxidative event (7,8,9,10,11). It is important to study the

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[1] Abbreviations used in this paper: AAPH, 2,2'-azobis(2-amidinopropane)dihydrochloride; DTT, dithiothreitol; Erk, extracellular signal-regulated kinase; GSH, reduced glutathione; GSSG, glutathione disulfide; IAA, iodoacetic acid; IEF, isoelectric focusing; NEM, N-ethylmaleimide.

[2] A note on nomenclature: GSH refers ONLY to the reduced form of glutathione. GSSG refers only to glutathione disulfide. Oxidized glutathione may refer to several forms of glutathione including GSSG, cysteine-glutathione disulfide, S-glutathiolated protein and S-nitrosoglutathione.

relationship between these two mechanisms, because both would affect the functioning of a wide range of proteins in cells during oxidative events.

Carbonic anhydrase III is a cytosolic protein that can be used as a model to study S-glutathiolation. It has two cysteine residues that are reactive to 1-chloro-2,4-dinitrobenzene (DTNB), alkylating agents, and oxidants (12,13). It has been shown *in vitro* to be S-glutathiolated by hydrogen peroxide (H_2O_2), diamide, GSSG and the xanthine/xanthine oxidase H_2O_2/superoxide-generating system (7,13,14). When GSH is not present to protect protein cysteines, xanthine/xanthine oxidase will also cause irreversible oxidation of carbonic anhydrase III *in vitro* (7). Irreversible oxidation is the formation of oxidized protein cysteine residues that are not reducible by thiol-disulfide exchange. These products may be either cysteine sulfinic acid or cysteine sulfonic acid (1,15,16,17). Both H_2O_2 and superoxide may have been responsible for the irreversible oxidation of carbonic anhydrase III by xanthine/xanthine oxidase (6), and so this process may be a general mechanism for damage to proteins by oxidants.

Differences in protein cysteine oxidation which occur with changing GSH levels may be responsible for redox regulation of cellular processes (18,19,20), although the mechanism for this has not been studied in detail. Cellular glutathione can affect such diverse processes as transcription (18,19), apoptosis (20), damage to DNA (21), cell division (20), proliferation of cancer cells (22), susceptibility to diseases (23,24), and enzyme activity (5). GSH is presumed to regulate these processes either by acting as an antioxidant, preventing oxidation of critical cysteines (25), or through formation of GSSG and subsequent S-thiolation of the proteins involved (26). Because GSSG levels in cells rarely reach levels necessary for S-glutathiolation, other mechanisms are necessary to explain S-glutathiolation *in vivo*.

Carbonic anhydrase III is in fact S-glutathiolated in hepatocytes after addition of exogenous oxidants such as diamide, menadione, and t-butyl hydroperoxide (7,14). In some cases S-glutathiolation occurs without increases in GSSG levels (8). When GSH is depleted from hepatocytes, menadione causes irreversible oxidation of carbonic anhydrase III (7). Thus, at normal GSH concentrations, reduced and S-glutathiolated forms of protein should predominate after oxidation. Irreversibly oxidized proteins may predominate when GSH concentrations are low. It has been proposed that tyrosine phosphatase is reversibly regulated by S-glutathiolation, and that S-glutathiolation prevents permanent inactivation of this protein by reactive oxygen species (27,28).

The effects of two different oxidants, 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) and H_2O_2, on carbonic anhydrase III are studied here. This study will determine the role of GSH concentration in the protection of protein cysteine residues from irreversible oxidation. In particular it will define at what concentrations GSH acts as a radical scavenger to prevent protein oxidation, and at what concentrations it participates in S-thiolation reactions. Differences between the two oxidants, AAPH and H_2O_2, are explored in this context. Evidence shows that proteins are good scavengers of oxidants relative to GSH, and that the major role of GSH in protection of proteins is in S-glutathiolation reactions.

Materials and Methods

Materials. l-Cysteine, dithiothreitol (DTT), reduced glutathione (GSH), glutathione disulfide (GSSG), and N-ethylmaleimide (NEM) were purchased from Sigma (St. Louis, MO). 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Trolox was purchased from Aldrich Chemical Company

(Milwaukee, WI). Ampholytes were purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Recombinant human erythrocyte carbonic anhydrase III was the generous gift of D.L. Silverman (University of Florida, Gainesville, FL).

Protein assay. Protein concentration was determined as described by Lowry et al. (29).

Isoelectric focusing (IEF) of Carbonic Anhydrase III. Purified carbonic anhydrase III was separated on horizontal slab gels (5.0% (acrylamide/2.7%Bis-acrylamide)/0.3% ampholyte pH 4.0-6.0/1.7% ampholyte pH 5.0-8.0) at 1500V and 1.1 watt/cm for 50 minutes at 4°C as previously described for rat liver carbonic anhydrase III (13). The reduced form of the human erythrocyte enzyme separated at pI 7.6, a slightly more basic pI than the rat liver enzyme (reduced form pI = 7.0) used previously in this laboratory (13). Gels were stained with Coomassie brilliant blue R-250 and air-dried.

Quantification of IEF gels. Gels were scanned and bands were quantified using Image Quant v3.3 (Molecular Dynamics Inc.). The extent of modification of carbonic anhydrase III was calculated by determining relative band densities within individual lanes. The fractional modification of carbonic anhydrase III was calculated from the following relationship:

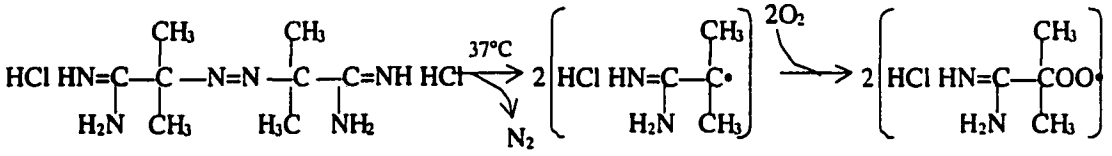
fractional modification = (density of band with 1 oxidized cysteine
+ 2 x density of bands with 2 oxidized cysteines)/density of all bands

Modification is reported in mole modified cysteine per mole of protein

Results

GSH protects carbonic anhydrase III from irreversible oxidation by peroxyradical generator AAPH. Purified carbonic anhydrase III is modified specifically on up to two cysteine residues following addition of 1-chloro-2,4-dinitrobenzene (DTNB), GSSG, diamide, t-butyl hydroperoxide, and xanthine/xanthine oxidase (7,12,13,14). It is modified in cells treated with diamide, menadione, and t-butyl hydroperoxide (7,14).

AAPH is a compound that generates alkyl peroxyradicals by thermal decomposition (Scheme 1). It has been used as a model for peroxyradical-mediated oxidation both *in vitro* and *in vivo* (30-32). AAPH splits homolytically at 37°C to form 2 moles of alkyl radical per mole of AAPH (33). These radicals then react quickly with molecular oxygen to form peroxyradicals, which can then react with sulfhydryls.



Scheme 1: Formation of peroxyradical from AAPH

Carbonic anhydrase III reaction was analyzed by IEF. In IEF gels, migration of a protein is based upon the pI of the protein. Addition of negative charge to the protein causes changes in migration of the protein toward the acidic end of the gel. When carbonic anhydrase III is incubated with AAPH more acidic bands appear compared to the unreacted protein (Figure 1, lane 2). This shows an oxidative reaction of carbonic anhydrase III with peroxyradicals that was not reversible by DTT (lane 3). The oxidized forms of the protein contain additional negative charge as measured by IEF and may be either cysteine sulfinic acid or cysteine sulfonic acid (1,15,16,17). When the reaction was carried out in the presence of 300 μM

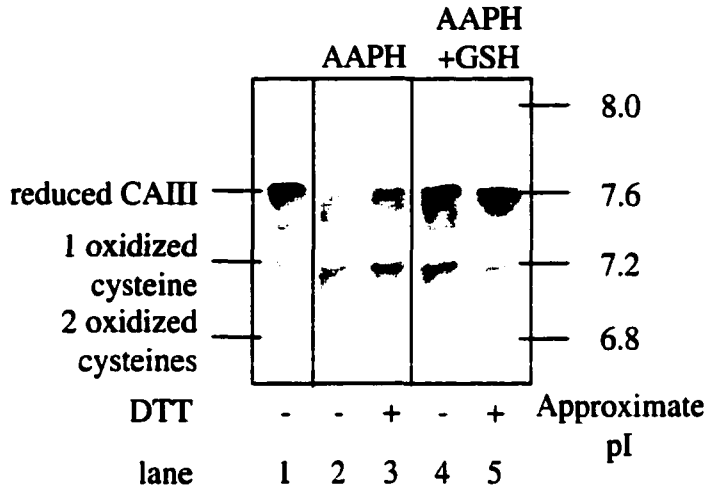


Figure 1. Oxidation of carbonic anhydrase III by AAPH in the presence of GSH.

Carbonic anhydrase III (30 μM) was incubated with 50 mM AAPH in the absence (lanes 2,3) or presence (lanes 4,5) of 300 μM GSH for 20 minutes in 50 mM sodium phosphate at pH 7.4 and 37°C. Reactions were stopped by incubating the reaction mixtures with 20 mM N-ethylmaleimide (NEM). Carbonic anhydrase III was reduced by incubating the reaction mixtures with 10 mM DTT for 20 minutes before addition of 20 mM NEM (lanes 3 and 5). IEF gel electrofocusing was described in Materials and Methods.

GSH, an acidic band also appeared (lane 4), indicating an oxidative event. This oxidation was almost entirely DTT-reversible (lane5). This GSH-mediated band shift is attributed to S-glutathiolation of carbonic anhydrase III cysteines because the reversibly oxidized carbonic anhydrase III is consistent with previous reports of S-glutathiolated carbonic anhydrase III (7,13).

AAPH treatment also produced a more basic form of the protein at pI 8.0 (lane 2) which was not DTT reversible (lane 3). The addition of positive charge to carbonic anhydrase III with oxidation has not been seen before. Peroxyradicals also react with carbon, so they are not entirely specific for sulfhydryl compounds (33,34), and this modification may represent the oxidation of another amino acid on carbonic anhydrase III. It was not possible to use SDS-PAGE to determine if there were any changes in molecular weight of carbonic anhydrase III caused by peptide chain breaks. The positive charge modification was not identified, nor was it used in quantification of damage to protein in this study. However, GSH does protect carbonic anhydrase III from this modification (Figure 1, lanes 4 and 5).

GSH may be preventing oxidative damage to carbonic anhydrase III by scavenging peroxyradicals in solution before they can react with the protein. It may also prevent damage to carbonic anhydrase III by forming disulfides with the protein, blocking the cysteine residues and preventing reactions with the free thiol. This second mechanism assumes that the cysteine residues are much more susceptible to damage than other amino acids, and without free sulfhydryls present, the overall reactivity of the protein is decreased. Figure 2 shows that either 500 or 100 μM Trolox, a vitamin E analogue, prevented modification of carbonic anhydrase III by AAPH (lanes 3 and 4) in comparison to carbonic anhydrase III that was incubated with AAPH alone (lane 2). If carbonic anhydrase III was treated with 20 mM

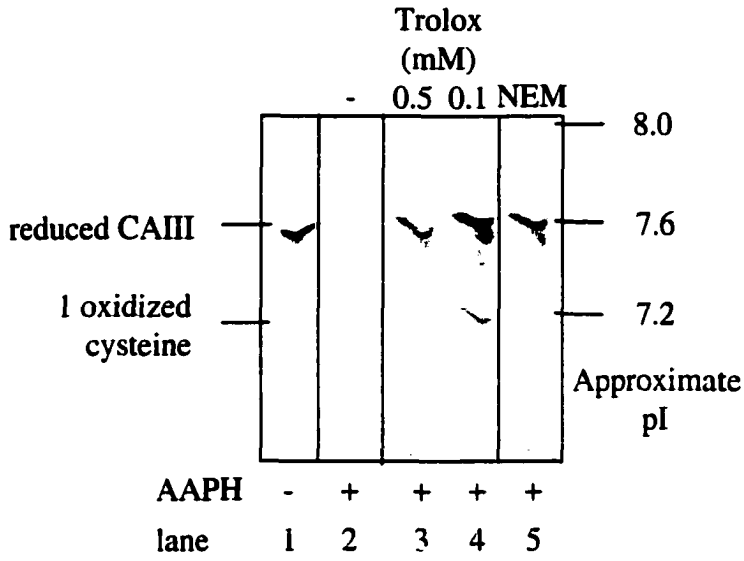


Figure 2: Protection of carbonic anhydrase III from AAPH-mediated oxidation.

Carbonic anhydrase III (30 μM) was incubated with AAPH for 20 minutes and then treated with 20 mM NEM to stop thiol modification. Lane 1, No AAPH treatment; lane 2, AAPH only; lanes 3 and 4, 500 μM and 100 μM Trolox, respectively, prior to AAPH addition; lane 5, 5 minute treatment with 20 mM NEM prior to AAPH addition.

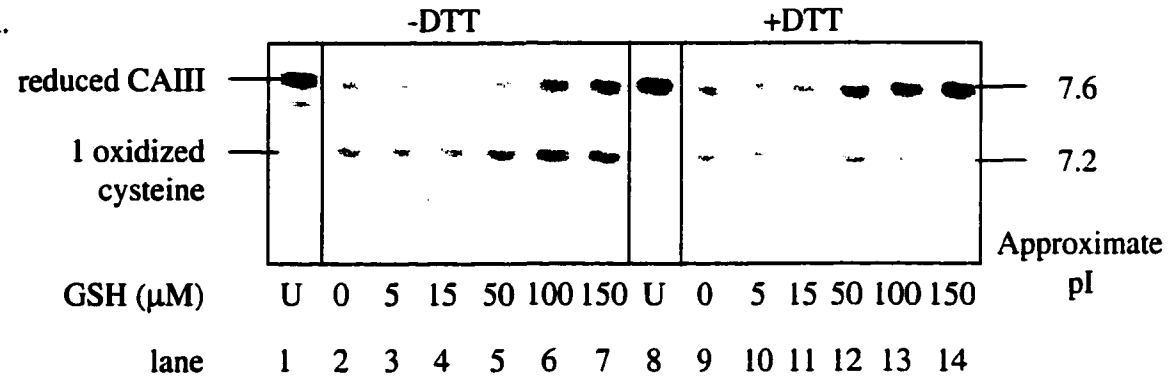
NEM prior to addition of AAPH (lane 5), the oxidative damage was also prevented. Thus, both Trolox and NEM protected against effects of AAPH. This protection included preventing the carbonic anhydrase III form that migrates at a more basic pI (pI= 8.0). This suggested that the cysteine residues were participating in propagation of radical species (2,25). Regardless, this experiment indicates that AAPH-mediated damage could be prevented both by scavenging peroxyradicals and by blocking sulfhydryl groups.

The ratio of GSH to protein is likely to be a critical factor in preventing oxidative damage to proteins. To test this, carbonic anhydrase III was oxidized with AAPH in the presence of varying concentrations of GSH. Figure 3A shows that AAPH oxidized carbonic anhydrase III in the absence of GSH (lane 2) and that this oxidation remained relatively constant as GSH concentrations were increased from 5 μM through 150 μM (lanes 3-7). Incubation with DTT reversed little of the damage occurring in the AAPH reaction when GSH was not present (lane 9), but as GSH concentrations increased (lanes 10-14) the modification became mainly reversible. Figure 3B is a plot of the calculated reversible and irreversible modification in this experiment. Total modification remained quite constant (approximately 60% of the protein was modified) and reversible modification increased with GSH concentration, while irreversible modification decreased. When GSH was in fivefold excess over carbonic anhydrase III concentration, irreversible oxidation was negligible. When GSH was further increased, protein modification decreased overall, thus 1.3 mM GSH decreased total modification to 5%, all of which was reversible (not shown). It is likely that this decrease is due to scavenging of peroxyradicals by GSH before they could react with the

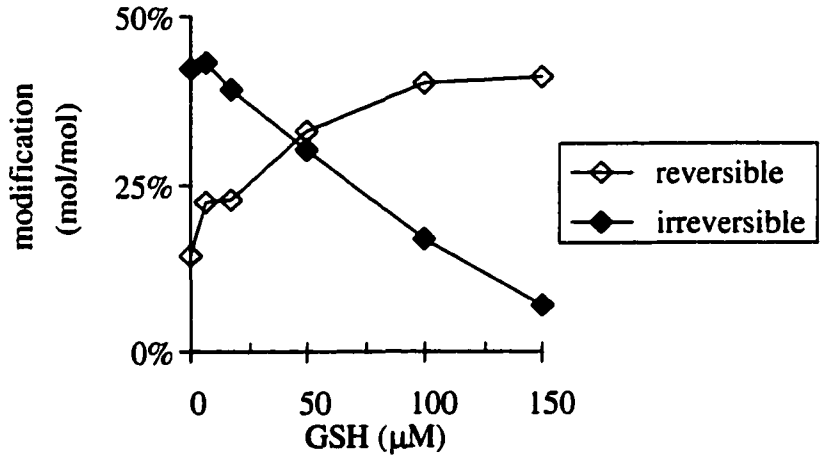
Figure 3. Effect of GSH concentration on carbonic anhydrase III modification by peroxyradicals.

Carbonic anhydrase III (30 μM) was incubated with 50 mM AAPH in the presence of varying concentrations of GSH for 20 minutes at 37°C in sodium phosphate buffer pH 7.4. Reactions were stopped by incubating the reaction mixtures with 20 mM N-ethylmaleimide (NEM). Carbonic anhydrase III was reduced by incubating the reaction mixtures with 10 mM DTT for 20 minutes before addition of 20 mM NEM (lanes 8-14). A. IEF separation of AAPH-treated carbonic anhydrase III. Concentrations of GSH are shown below each lane, U = no AAPH added. B. Analysis of reversible modification and irreversible modification dependence on GSH concentration. Fractional modification (modification) of carbonic anhydrase III was determined by analysis of the IEF separation in part A as described in Materials and Methods. Reversible modification is the difference in fractional modification between DTT-untreated and DTT-treated lanes. Irreversible modification is the fractional modification in DTT-treated lanes. C. Carbonic anhydrase III (30 μM) was incubated with either 50 mM AAPH and 150 μM GSH or with 75 μM GSSG at pH 7.4 and at 37°C for 20 minutes. Reversible modification was determined as described in part B of this figure.

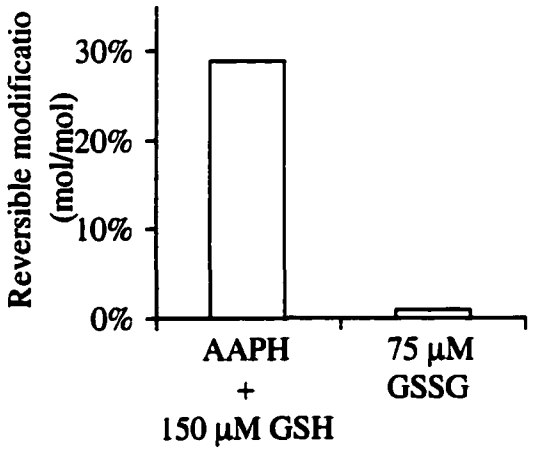
A.


-DTT
+DTT
reduced CAIII
1 oxidized cysteine
7.6
7.2
Approximate pI
GSH (μM) U 0 5 15 50 100 150 U 0 5 15 50 100 150
lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14

B.


50%
25%
0%
modification (mol/mol)
0
50
100
150
GSH (μM)
reversible
irreversible

C.


Reversible modificatio (mol/mol)
30%
20%
10%
0%
AAPH + 150 μM GSH
75 μM GSSG

protein.

It has been suggested that reactive oxygen species cause S-glutathiolation of proteins by reacting with two GSH molecules to form GSSG. GSSG could then react with protein cysteine by thiol-disulfide exchange to form the S-glutathiolated protein (26). Figure 3C shows data relevant to this idea. When carbonic anhydrase III was incubated with 75 μM GSSG (the maximum that could be produced by oxidation of 150 μM GSH), carbonic anhydrase III was not significantly modified. This is in comparison to 30% modification in the reaction of AAPH with carbonic anhydrase III in the presence of 150 μM GSH. Thus, GSSG cannot be an intermediate in S-glutathiolation of carbonic anhydrase III by AAPH. This supports previous reports that oxidants react directly with proteins or GSH to form an oxidized intermediate, and that this intermediate in turn reacts to form the S-glutathiolated product (6,8).

When carbonic anhydrase III concentration was varied from 30 μM to 240 μM while holding GSH concentration at 150 μM, with AAPH treatment, it was found that reversible modification increased in proportion to carbonic anhydrase III concentration (Figure 4). Irreversible modification was negligible in this experiment (not shown). S-glutathiolation was about 30% at 30 μM carbonic anhydrase III and decreased only slightly to about 20% modification when carbonic anhydrase concentration was 240 μM. Since the amount of S-glutathiolated carbonic anhydrase III increases with carbonic anhydrase III concentration, it appears that carbonic anhydrase III is trapping peroxyradicals very efficiently. When carbonic anhydrase III concentration is 240 μM, there is 40 μM of protein bound glutathione, accounting for 25% of the total GSH available. S-glutathiolated protein therefore accounts

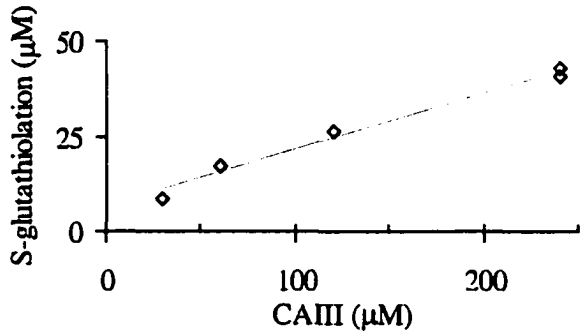
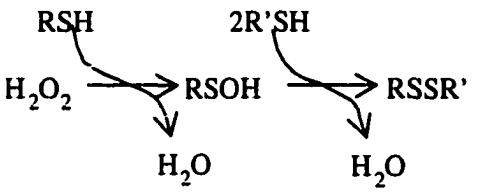


Figure 4. Carbonic anhydrase III concentration effects on AAPH reaction.

AAPH (50 mM) was incubated with 30 μM to 240 μM carbonic anhydrase III in the presence of 150 μM GSH at 37°C for 20 minutes. Reactions were stopped by incubating the reaction mixtures with 20 mM N-ethylmaleimide (NEM). Carbonic anhydrase III was reduced by incubation of the reaction mixtures with 10 mM DTT for 20 minutes before addition of 20 mM NEM (lanes 3 and 5). Carbonic anhydrase III was then separated by IEF as described in Materials and Methods. Fractional modification of carbonic anhydrase III was determined by analysis of the IEF separation as described in Materials and Methods. Reversible modification is the difference in fractional modification between DTT-untreated and DTT-treated lanes. Irreversible modification is the fractional modification in DTT-treated lanes.

for a significant fraction of oxidized glutathione in this experiment. This shows that carbonic anhydrase III is an efficient scavenger of AAPH generated radicals via S-glutathiolation.

GSH protects carbonic anhydrase III from irreversible oxidation by H_2O_2. H_2O_2 will react directly with sulfhydryls to form sulfenic acid (scheme 2) (25). The sulfenic acid will then react rapidly with free thiols to form a disulfide. In the absence of available thiols,



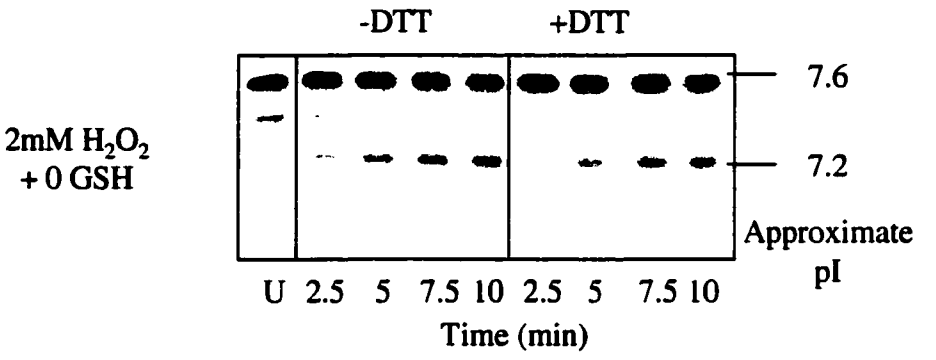
Scheme 2: Reactions of H_2O_2 with RSH.

sulfenic acid will continue to react with H_2O_2 to form sulfinic acid and sulfonic acid (15,16). When carbonic anhydrase III was incubated with H_2O_2 for less than 10 minutes (Figure 5A) an acidic band (pI = 7.2) appeared, analogous to that produced by the AAPH reaction (Figures 1,3). In the absence of GSH (Figure 5A.), the modification was not reversible with DTT treatment (compare right and left half of the same gel). Unlike AAPH, H_2O_2 did not produce a more basic protein band at pI=8.0 (not shown). This is because H_2O_2 oxidation is probably more specific for cysteine than is oxidation generated by AAPH. When GSH was added to the reaction (Figures 5B, 5C), the oxidative modification to carbonic anhydrase III was more pronounced and completely reversible (compare right and left half of each gel). Figure 5D quantifies the oxidatively modified carbonic anhydrase III as shown in parts A, B, and C of this figure. As GSH concentration increased, reversible modification (open symbols) increased and irreversible modification (closed symbols) decreased. This indicates

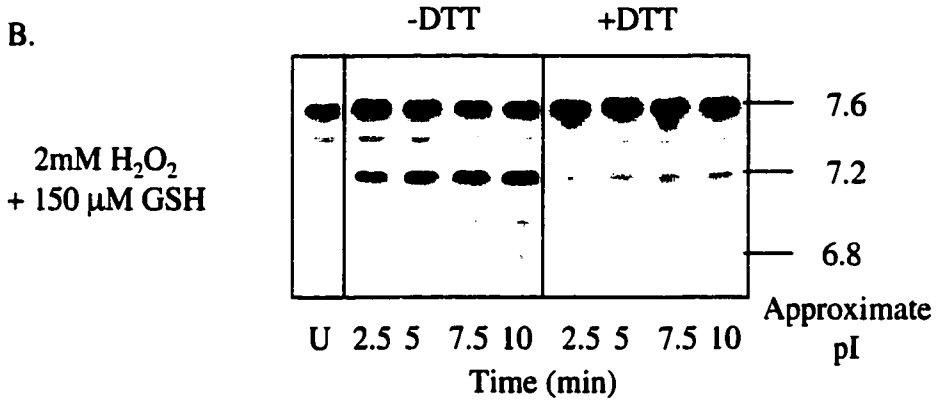
Figure 5. Oxidation of carbonic anhydrase III with H_2O_2 and GSH.

Carbonic anhydrase III (10 μM) with the indicated concentration of GSH was incubated with 2 mM H_2O_2 at 37°C in 20 mM sodium phosphate buffer pH 7.4. Reactions were stopped at the indicated time by incubating the reaction mixtures with 20 mM N-ethylmaleimide (NEM). Carbonic anhydrase III was reduced (right half of each gel) by incubating the reaction mixtures with 10 mM DTT for 20 minutes before addition of 20 mM NEM. IEF separations were performed as described in Materials and Methods. A. Carbonic anhydrase III + H_2O_2 without addition of GSH. B. Carbonic anhydrase III + 150 μM GSH + H_2O_2. C. Carbonic anhydrase III + 450 μM GSH + H_2O_2. D. Analysis of the reaction between carbonic anhydrase III, GSH and H_2O_2. Fractional modification of carbonic anhydrase III was determined by analysis of the IEF separations in parts A, B, and C of this figure as described in Materials and Methods. Reversible modification is the difference in fractional modification between DTT-untreated and DTT-treated lanes. Irreversible modification is the fractional modification in DTT-treated lanes.

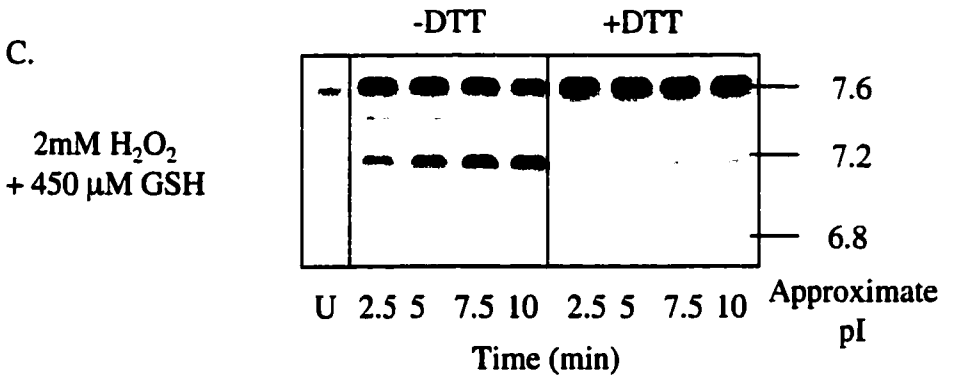
A.


-DTT
+DTT
2mM H_2O_2
+ 0 GSH
7.6
7.2
Approximate
pI
U 2.5 5 7.5 10 2.5 5 7.5 10
Time (min)

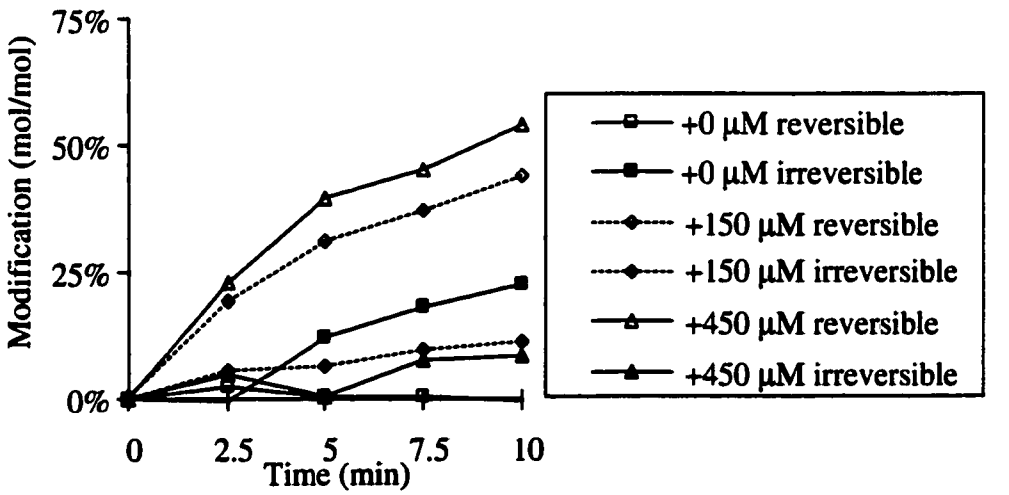
B.


-DTT
+DTT
2mM H_2O_2
+ 150 µM GSH
7.6
7.2
6.8
Approximate
pI
U 2.5 5 7.5 10 2.5 5 7.5 10
Time (min)

C.


-DTT
+DTT
2mM H_2O_2
+ 450 µM GSH
7.6
7.2
6.8
Approximate
pI
U 2.5 5 7.5 10 2.5 5 7.5 10
Time (min)

D.


75%
50%
25%
0%
Modification (mol/mol)
0
2.5
5
7.5
10
Time (min)
+0 µM reversible
+0 µM irreversible
+150 µM reversible
+150 µM irreversible
+450 µM reversible
+450 µM irreversible

that reversible modification of carbonic anhydrase III is a fast reaction in comparison to irreversible modification and is favored when substrate (i.e. GSH) is available. Total modification (reversible + irreversible) at 150 μM or 450 μM GSH was approximately double the total modification when GSH was absent. Again this suggests that S-glutathiolation is fast in comparison to irreversible oxidation.

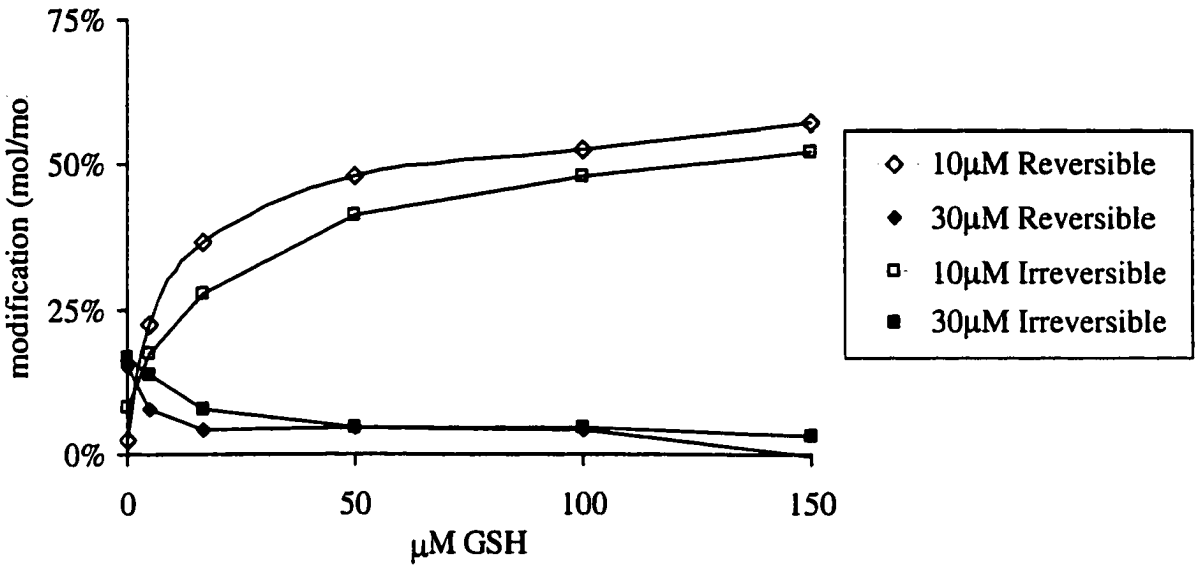
The concentration dependence for the reaction between carbonic anhydrase III, GSH and H_2O_2 was determined at two concentrations (10 μM and 30 μM) of carbonic anhydrase III. Figure 6A shows that reversible modification (open symbols) reached maximal levels at 50 μM GSH at both protein concentrations. Carbonic anhydrase III was also completely protected from irreversible oxidation (closed symbols) by 50 μM GSH. At GSH concentrations below 50 μM, irreversible oxidation of carbonic anhydrase III was dependent on carbonic anhydrase III concentration. When carbonic anhydrase III concentration was 30 μM, H_2O_2 caused more irreversible oxidation at 5 μM and 15 μM GSH than when carbonic anhydrase III concentration was 10 μM. As noted above, 50 μM GSH completely protected 30 μM carbonic anhydrase III from irreversible oxidation. Thus, protection of proteins by GSH was most efficient when GSH:protein molar ratios approached one. When 5 μM GSH was present, S-glutathiolated protein accounted for 50% and 100% of the GSH available in reactions of H_2O_2 with 10 μM and 30 μM carbonic anhydrase III, respectively. Thus, when GSH is limiting, S-glutathiolation is likely to be the primary function of GSH in protection of carbonic anhydrase III from irreversible oxidation by H_2O_2.

At 1.3 mM GSH, 50% of the carbonic anhydrase III was S-glutathiolated, and none was irreversibly oxidized (data not shown). Carbonic anhydrase III was modified by H_2O_2

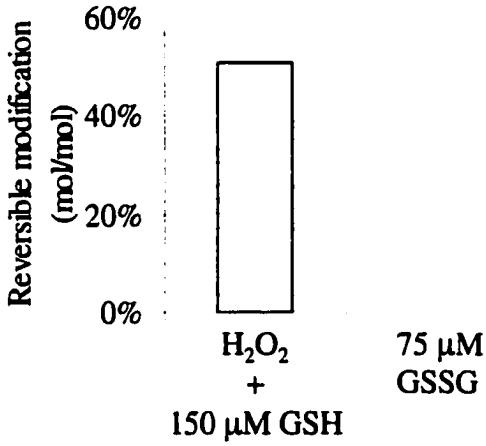
Figure 6. Effect of GSH concentration on carbonic anhydrase III modification by H_2O_2.

Carbonic anhydrase III (10 μM or 30 μM) with the indicated concentrations of GSH was incubated with 2 mM H_2O_2 for 10 minutes at 37°C and at pH 7.4. Reactions were stopped by incubating the reaction mixtures with 20 mM N-ethylmaleimide (NEM). Carbonic anhydrase III was reduced by incubation of the reaction mixtures with 10 mM DTT for 20 minutes before addition of 20 mM NEM. Carbonic anhydrase III was then separated by IEF as detailed in Materials and Methods. Fractional modification of carbonic anhydrase III was determined by analysis of the IEF separation as described in Materials and Methods. Reversible modification is the difference in fractional modification between DTT-untreated and DTT-treated lanes. Irreversible modification is the fractional modification in DTT-treated lanes. B. Carbonic anhydrase III (30 μM) was incubated with either 2 mM H_2O_2 and 150 μM GSH or with 75 μM GSSG for 10 minutes at 37°C and at pH 7.4. Reactions were stopped and protein was reduced as in part A. Modification was analyzed as in part A of this figure.

A.


75%
50%
25%
0%
modification (mol/mo
0
50
100
150
µM GSH
10µM Reversible
30µM Reversible
10µM Irreversible
30µM Irreversible

B.


Reversible modification (mol/mol)
60%
40%
20%
0%
H2O2 + 150 µM GSH
75 µM GSSG

even when GSH concentrations were 130 times greater than the protein concentration. This confirms that carbonic anhydrase III is a good scavenger of H_2O_2 compared to GSH.

GSSG formation could not account for the extent of S-glutathiolation seen in the reaction of carbonic anhydrase III with AAPH (Figure 3C). Similarly, the reaction between carbonic anhydrase III and 75 μM GSSG produces little modification in comparison to that of carbonic anhydrase III, 150 μM GSH, and H_2O_2 (Figure 6B). GSSG clearly is not an intermediate in the S-glutathiolation of carbonic anhydrase III by H_2O_2. As with AAPH, it is likely that H_2O_2 reacts with either carbonic anhydrase III or GSH, which in turn reacts to form S-glutathiolated protein.

GSH has been shown to be the major thiol participating in S-thiolation reactions in cells (8). This may be because of a special affinity of protein S-thiolation sites for GSH, or because of the relatively high concentration of GSH (35) compared to other low molecular weight thiols in cells. We therefore studied whether cysteine would also protect carbonic anhydrase III from oxidative damage by H_2O_2. When carbonic anhydrase III was incubated with H_2O_2, 30% of the protein was modified by a single negative charge (Figure 7, second bar). Cysteine prevented this modification by H_2O_2 as shown by its inhibition of negative charge addition (third bar). In order to be able to detect the neutrally charged S-cysteylated carbonic anhydrase III, the protein was reacted with the negatively charged alkylating agent iodoacetic acid (IAA). S-cysteylation would appear in this assay as an inhibition of IAA derivitization, while irreversible oxidation and IAA derivitization would be indistinguishable (36). When carbonic anhydrase III is reacted with IAA , it is modified with a negative charge on 1 site on 100% of the protein (fourth bar), which agrees with previously published

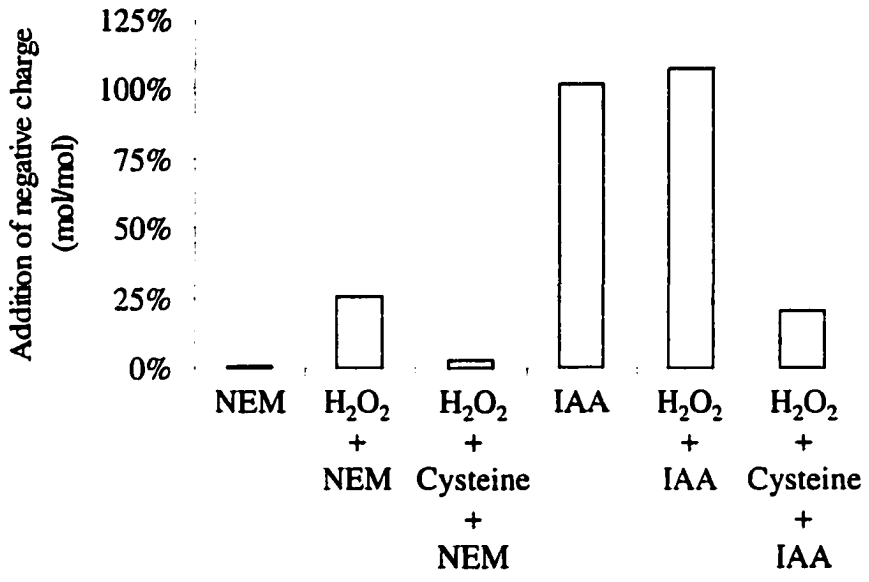


Figure 7. Protection of carbonic anhydrase III from H_2O_2 oxidation by cysteine.

Carbonic anhydrase III (10 μM) was incubated with 2 mM H_2O_2 in the absence and presence of 150 μM cysteine at 37°C and at pH 7.4 for 10 minutes. The reactions were stopped with either 10 mM IAA or 10 mM NEM as indicated. IEF was performed as described in Materials and Methods. Addition of negative charge was then calculated as the fractional modification as described in Materials and Methods.

reports of rat liver carbonic anhydrase III (36). If carbonic anhydrase III is incubated with H_2O_2 before IAA derivitization, there is a modest increase in negatively charged modification (fifth bar). This small increase indicates that little of the modification caused by H_2O_2 occurred on the thiol which is does not react with IAA. Only 30% of the protein is modified with a negative charge when cysteine is present during the reaction of H_2O_2 with carbonic anhydrase III (sixth bar), indicating that more than 70% of the protein is S-cysteylated. Therefore GSH may participate in S-thiolation reactions largely due to its concentration in cells relative to the concentrations of other low molecular weight thiols.

Discussion:

From the data presented here three distinct mechanisms for protection of protein cysteines can be proposed (Figure 8): 1) The oxidant reacts first with protein cysteine to form an activated protein cysteine, either protein thiyl radical, protein cysteine thioperoxide, protein cysteine thioperoxyradical, or protein cysteine sulfenic acid (5,18,25,37). This activated cysteine then reacts with GSH to form the S-glutathiolated protein (Reaction 1). 2) The oxidant reacts first with GSH to form an activated form of glutathione, which then reacts with protein cysteine to form S-glutathiolated protein (Reaction 2). 3) The oxidant reacts first with GSH to form an activated form of glutathione, which then reacts with another molecule of GSH to form GSSG (Reaction 3). In reaction 4, the oxidant reacts with the protein to form an activated intermediate that, in the absence of GSH, leads to irreversible oxidation.

Oxidation of carbonic anhydrase III with either AAPH or H_2O_2 produced considerable

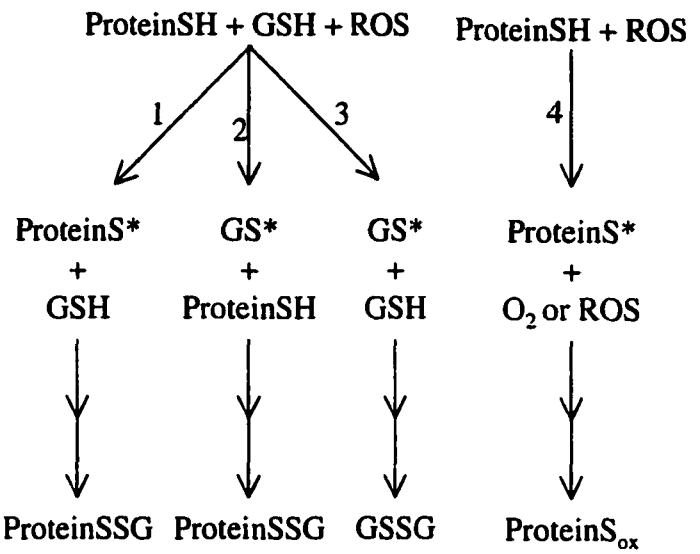


Figure 8: Mechanisms for Protection of Protein Cysteine (ProteinSH) from Irreversible Oxidation by Reactive Oxygen Species (ROS).

Reaction 1: Reactive oxygen species (ROS) react with the protein to form an activated intermediate, ProteinS* (e.g. thiyl radical or cysteine sulfenic acid), which then reacts with GSH to form S-glutathiolated protein, ProteinSSG. Reaction 2: ROS react with GSH to form an activated intermediate, GS*, which then reacts with ProteinSH to form S-glutathiolated protein. Reaction 3: ROS react with GSH to form an activated intermediate that then reacts with GSH to form GSSG. Reaction 4: ROS react with the protein in the absence of GSH to form an activated intermediate which can then react with molecular oxygen to form irreversibly oxidized protein, $ProteinS_{ox}$.

irreversible oxidation in the absence of GSH, which validates reaction 4.

Multiple acidic bands are generated on the IEF separation which are not reducible by DTT treatment (Figure 1). These bands also occur in oxidation of carbonic anhydrase III by other mechanisms (7,36). Reactions between thiols and reactive oxygen species may result in formation of sulfinic acid or sulfonic acid (1,15,16,17), both of which are negatively charged sulfur compounds. It therefore seems likely that AAPH and H_2O_2 treatment of carbonic anhydrase III results in the formation of protein cysteine sulfinic or sulfonic acid.

GSH prevented irreversible oxidation by AAPH or H_2O_2 (Figure 8, Reactions 1,2 and 3) and simultaneously participated in S-glutathiolation of carbonic anhydrase III (reactions 1 and 2). The protection of carbonic anhydrase III and formation of S-glutathiolated protein was dependent on the concentration of GSH. Additionally, as S-glutathiolation increased, irreversible oxidation decreased in a GSH-dependent manner. This suggests that S-glutathiolation is linked to prevention of irreversible oxidation (5-7).

At lower GSH concentrations, reactions 1 and/or 2 are important, since S-glutathiolated protein is a measured endpoint in both reactions. The increase in total protein modification at low GSH concentrations suggests either that reaction 2 is an important mechanism, or that formation of irreversible oxidation products (reaction 4) is slower than the S-glutathiolation reaction (i.e. the second step) in reaction 1. Because formation of cysteine sulfinic acid and cysteine sulfonic acid require multiple oxidation events, this supposition is reasonable. S-glutathiolation of carbonic anhydrase III is 50% mole/mole at GSH:protein ratios of 130:1 in the H_2O_2 reaction and 30% mole/mole when GSH:protein ratios vary from 5:1 through 1:1.5 in the AAPH system. This indicates that reaction 1, which postulates interaction of oxidant with the protein, is important. The fact that reaction 4

occurs at all is further proof that the protein must react directly with the oxidant as it does in the first step of reaction 1.

GSSG formation is not an important mechanism of S-glutathiolation in AAPH or H_2O_2-mediated oxidation, and so reaction 3 would be more important only at very high GSH:protein ratios. In the AAPH reaction, GSH:protein ratios of 130:1 produced 5% S-glutathiolation, while the maximal S-glutathiolation (at lower GSH concentrations) was 30%. In contrast, 130:1 GSH:protein ratio in the H_2O_2 reaction produced 50% S-glutathiolation versus 60% at maximal levels. This shows that carbonic anhydrase III is a more efficient scavenger of H_2O_2 than it is of AAPH-generated peroxyradicals. However, because GSH:protein thiol ratios are close to 1:1 by most estimates, and certainly do not reach 130:1, the significance of very high GSH:protein ratios is questionable.

The reaction of H_2O_2 with carbonic anhydrase III and cysteine suggests that cysteine is as effective as an S-thiolating agent as GSH when carbonic anhydrase III is the protein substrate. If carbonic anhydrase III is typical of cytosolic proteins with reactive cysteines, then the predominance of S-glutathiolated proteins in oxidized cells is likely the result of the greater concentration of GSH in cells (8). Since carbonic anhydrase III does not appear to have a binding site for GSH, this result should be relevant to many surface-exposed cysteine sites on proteins.

In conclusion, GSH participates in oxidative reactions with proteins to form mixed disulfides. S-glutathiolation appears to occur by reaction of either protein cysteine or GSH with the reactive oxygen species, which then reacts to form S-glutathiolated protein. The reaction between cysteine and carbonic anhydrase III argues against the necessity for specific interactions between protein and GSH for S-glutathiolation. GSSG formation is not a

significant mechanism for S-glutathiolation in either of the systems studied. The formation of S-glutathiolated proteins prevents formation of irreversibly oxidized species *in vitro* at GSH:protein ratios of 1:1 up to at least 5:1 and in the case of H_2O_2, up to at least 130:1. Direct scavenging of reactive oxygen species by GSH is likely to be important at very high GSH:protein ratios although GSH seemed a more effective scavenger of AAPH than of H_2O_2. Because GSH:protein ratios in cells are more likely to be closer to 1:1 than 130:1 (8), the formation of S-glutathiolated protein is probably the physiological mechanism for protection of protein sulfhydryls against permanent inactivation by the formation of irreversibly oxidized species. Furthermore, the reaction mechanisms postulated here (Figure 8, reactions 1 and 2) suggest that regulation of proteins can occur through S-glutathiolation without the necessity of forming GSSG as an intermediate. This makes S-glutathiolation a plausible initial event in the activation of signaling cascades by oxidants. While the prevention of irreversible oxidation by S-glutathiolation has obvious value in preserving the function of cysteine-containing proteins, irreversible oxidation of cysteines may contribute to degeneration of signaling seen in aging, cancer and other disease states (38-40).

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CHAPTER II:

S-GLUTATHIOLATION OF H-RAS *IN VITRO* AND *IN VIVO*

A paper to be submitted to the Journal of Biological Chemistry

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Abstract

The oxidation of H-Ras thiols was studied in both the pure protein and in NIH-3T3 cells. Purified H-Ras was found to be modified by thiol oxidants including hydrogen peroxide (H_2O_2), S-nitrosoglutathione, diamide, GSSG, and cystamine, producing up to four modified forms. Since H-Ras is known to contain four reactive sulfhydryls, these results suggest that all four cysteines can react with a variety of oxidants. Both S-nitrosylated and S-glutathiolated forms of H-Ras were identified by separation on electrofocusing gels. S-nitrosoglutathione appears to cause S-nitrosylation of H-Ras on four cysteine residues as determined by IEF. Reduced glutathione (GSH) mediates S-glutathiolation by H_2O_2 on at least one cysteine of purified H-Ras as measured by IEF and HPLC. Either GSSG or diamide + GSH appears to S-glutathiolate at least two cysteine residues of purified H-Ras. Iodoacetic acid reacts with three cysteine residues.

In intact NIH-3T3 cells, H-Ras was S-thiolated by diamide. In cells expressing a C118S mutant or a C181S/C184S double mutant, H-Ras was also S-thiolated. These results

suggest that H-Ras can be oxidatively modified on multiple thiols *in vivo* and that at least one of these thiols is normally lipid modified. Thus, cellular oxidative stress may lead to competition between oxidant and lipid for reaction with H-Ras thiols. This might alter the membrane association and activity of H-Ras.

Introduction

H-Ras, a small monomeric guanine nucleotide binding protein (G-protein) is essential in most cells for proliferation and differentiation (1). It participates in the well-studied extracellular signal-regulated kinase (Erk)[1] pathway and has been implicated in relaying oxidative signals through this pathway (2-5). Studies have shown activation of Erk and other components of the Erk pathway when cells were treated with H_2O_2, NO, superoxide, sodium nitroprusside, and S-nitroso-N-acetylpenicillamine. The oxidative response of the Erk pathway may be a direct result of changes in the GTP/GDP ratio of H-Ras, altered interaction of H-Ras with an effector molecule, modification of one of the effector molecules on the interaction with H-Ras, or process that leaves H-Ras unchanged (3,4,6,7).

Cell culture experiments using Jurkat cells showed that, upon treatment of the cells with nitric oxide (NO) related compounds, the Erks were activated and the GTP/GDP ratio of the H-Ras protein was altered (3,6). *In vitro* results showed that an NO-treated truncated mutant of H-Ras lacking the three c-terminal cysteine residues was specifically modified by

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[1] Abbreviations used in this paper: DTT, dithiothreitol; Erk, extracellular signal-regulated kinase; GSH, reduced glutathione; GSSG, glutathione disulfide; IAA, iodoacetic acid; IAM, iodoacetamide; IEF, isoelectric focusing; NEM, N-ethylmaleimide.

S-nitrosylation on Cys118 (6). The authors suggest that a direct modification of Cys118 by NO may be responsible for the activation of H-Ras in cells treated by NO gas, and that S-nitrosylation of H-Ras was at least partially responsible for the proliferative effects of NO in cells. Because Cys118 resides on a loop that has contact with the guanine nucleotide, modification of this cysteine may directly affect the bound GTP/GDP ratio of H-Ras, thus changing its activity (8).

H-Ras possesses three other cysteine residues that could be modified *in vivo*, the effects of which would likely oppose that suggested for the modification of Cys118. For example, it has been shown that NO-related oxidants can prevent palmitoylation of some proteins in cells (9). Since Cys181 and Cys184 of H-Ras are palmitoylated, a transient modification *in vivo*, a significant portion of these residues would potentially be available for modification (10,11). Oxidation of one or both of these residues could alter membrane association of H-Ras and thus affect the signal transduction properties of the protein. Mutagenesis studies have confirmed that these residues are essential for transformation of cells by an otherwise oncogenic H-Ras mutant form (11).

Cys186 of H-Ras has been shown to be farnesylated via a thioether linkage. Farnesylation of the protein allows it to associate with cellular membranes (10,12-14). Although the H-Ras protein is thought to remain farnesylated for the lifetime of the protein, a significant fraction of these cysteines are available in most cells (11,15). It is possible that oxidative modification of this residue occurs in cells. Oxidation of this residue might inactivate H-Ras, since farnesylation and membrane association is thought to be essential for its function (10,12-14).

Cells are continually exposed to oxidative conditions that can potentially damage many of their components (16,17). S-glutathiolation of proteins is directly related to the oxidation of proteins in cells (17). S-glutathiolation is a two-electron oxidation of protein cysteine and free reduced glutathione (GSH)[2] to a disulfide of protein cysteine-glutathione disulfide. It can occur when a protein cysteine is oxidized and subsequently reacts with GSH to form the mixed disulfide (18,19). The S-glutathiolated protein may be reduced back to its original thiol form by the dithiol protein glutaredoxin (20). The availability of GSH to form S-glutathiolated proteins may be linked to the prevention of irreversible damage of these sulfhydryls (18,19, see chapter I).

If H-Ras is found to be S-glutathiolated or otherwise oxidatively modified, it would be an important step in understanding primary mechanisms of oxidative regulation of signal transduction pathways. This paper provides evidence that H-Ras may be oxidatively modified on four separate cysteine residues *in vitro*. We further show that while S-nitrosylation may modify all four of these residues, S-glutathiolation occurs primarily on two of four possible reactive cysteine residues. We also provide evidence that one or more sites are available to S-glutathiolation *in vivo*, and that at least one of these sites is a cysteine normally required for lipidation of H-Ras.

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[2] A note on nomenclature: GSH refers ONLY to the reduced form of glutathione. GSSG refers only to glutathione disulfide. Oxidized glutathione may refer to several forms of glutathione including GSSG, cysteine-glutathione disulfide, S-glutathiolated protein and S-nitrosoglutathione.

Materials and Methods

Materials. Iodoacetic acid (IAA), iodoacetamide (IAM), N-ethylmaleimide (NEM), reduced glutathione (GSH), dithiothreitol (DTT), glutathione disulfide (GSSG), rat anti-mouse alkaline phosphatase-conjugated antibody and goat anti-rat alkaline phosphatase-conjugated antibody were from Sigma (St. Louis, MO). Cell culture reagents were purchased from GIBCO-BRL (Gaithersburg, MD) with the exception of bovine calf serum (BCS), which was purchased from HyClone (Logan, UT). Ampholytes were purchased from Amersham-Pharmacia, Inc (Piscataway, NJ). Recombinant human H-Ras and agarose-conjugated and soluble Ab-1 were purchased from Calbiochem-Novabiochem (San Diego, CA). Monoclonal Ab 146-03E4 (Ab 146) was purchased from Quality Biotech (Camden, NJ).

Protein assay. Protein concentration was determined as described by Lowry et. al. (25).

Preparation of H-Ras Protein. Purified wildtype H-Ras (3 mg/ml) was completely reduced with 10 mM DTT for 30 minutes at room temperature. The protein was then dialyzed for 24 hours at 4°C against 20 mM Tris-Cl pH 8.0, containing 500 μM DTT. DTT was included in dialysis buffer to prevent progressive oxidation of H-Ras that occurred in the absence of DTT (data not shown). Dialyzed protein (approximately 1mg/ml) was stored for less than one week at 4°C before use.

Preparation of S-nitrosothiols. S-nitrosoglutathione was prepared as previously described in (21) and used immediately after preparation. Briefly, 220 μl each of 220 mM GSH and 220 mM sodium nitrite were mixed with 25 μl of 4.0 N HCl and incubated in the dark at room temperature for 10 minutes. The solution was then neutralized with 25 μl of 4.0

N NaOH to give a final concentration of approximately 100 mM S-nitrosoglutathione. The final concentration was calculated from absorbance at 334 nm using the extinction coefficient 767 M^{-1} cm^{-1} (24)

Isoelectric focusing (IEF) of H-Ras. Purified H-Ras was separated on horizontal slab gels (5.0% acrylamide/2.7%Bis-acrylamide/1% ampholyte pH 3.5-5.0/1% ampholyte pH 4.0-6.0) at 1500V and 0.5mAmp/cm^2 for 50 minutes. Approximately 1 μg of H-Ras was applied to each lane. Gels were stained with Coomassie Brilliant Blue as previously described (21).

Isoelectric focusing/Western Blot analysis of H-Ras. H-Ras was separated by IEF as previously described (21) and transferred to Immobilon-P (PVDF) (Millipore, Inc, Bedford, MA) membrane using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA) according to the recommendation of the manufacturer. H-Ras was visualized with the Ab 146 primary antibody (1:3000 dilution) and rat antimouse alkaline phosphatase secondary Ab (1:10000 dilution) with the exception of the truncated mutant (Δ167-190) which was visualized using Ab-1 as the primary antibody (1:3,000 dilution) and goat anti-rat secondary antibody (1:10,000 dilution). Bands were visualized with p-nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indoyl phosphate (BCIP).

Quantification of IEF gels. Gels were scanned and bands were quantified using Image Quant v3.3 (Molecular Dynamics Inc.). The extent of modification of H-Ras was calculated by determining relative band densities within individual lanes. The fractional modification of H-Ras was calculated from the following relationship:

fractional modification = (density of band with 1 oxidized cysteine
+ 2 x density of bands with 2 oxidized cysteines
+ 3 x density of bands with 3 oxidized cysteines)/density of all bands

Modification is reported in mole modified cysteine per mole of protein

Modification of H-Ras in soluble extracts of E. coli overexpressing H-Ras. As described by Ji. et. al. (21), *E. coli* strain JM 105 containing the gene for H-Ras wildtype, C181S/C184S or truncated (Δ167-190) mutant donated by S. Campbell (University of North Carolina, Chapel Hill, NC) inserted in a pAT vector (22) were grown at 37°C in Luria broth to an OD of 1.2-1.5 (at 600nm) and induced by addition of isopropyl,β-D-thiogalactopyranoside for 3 hours. The cells were then centrifuged at 16,000 x g for 10 minutes. The pellet was washed with 20 mM Tris-HCl buffer (pH 7.2), 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM phenylmethyl sulfonyl fluoride (PMSF). After centrifugation at 16,000g for 10 minutes, cells were resuspended to 0.2 g cell paste/ml in 20 mM β-glycerophosphate-HCl (pH 7.0) and sonicated for 30 seconds. The soluble fraction was obtained by ultracentrifugation in a Beckman Airfuge at 160,000 x g for 30 minutes.

Cell Culture. NIH-3T3 fibroblasts were obtained from American Type Culture Collection (ATCC) and cultured according to their recommendations (10% CO_2, Dulbecco's Modified Eagle Medium (DMEM) + 2mM Glutamine + 0.2mM Sodium Pyruvate + 10% BCS + 100 units/ml each of Penicillin and Streptomycin). NIH-3T3 cells overexpressing wildtype and C118S H-Ras were generous gifts from L.A. Quilliam (Indiana University School of Medicine, Indianapolis, IN) and were cultured according to ATCC recommendations for NIH-3T3 cells. NIH-3T3 cells overexpressing C181S/C184S H-Ras were the generous gift of B.M. Willumsen (University of Copenhagen, Copenhagen, Denmark.) and were cultured according to ATCC recommendations for NIH-3T3 cells.

Cellular experiments. NIH-3T3 cells were grown to confluence (assessed visually, approximately 1-2 x 10^6 cells/35 mm plate) and the medium was changed. Experiments were

started 24 hours later. Experiments were conducted in phosphate buffered saline (PBS) to avoid any oxidative artifacts associated with interactions between DMEM and added oxidants. PBS was added to cells 5 minutes prior to the addition of oxidant to avoid oxidative artifacts associated with change of medium.

Analysis of Cellular Low Molecular weight thiols. NIH-3T3 cells were rinsed twice with ice cold PBS and lysed with 10% perchloric acid. Plates were scraped and the soluble and precipitated materials were collected and stored at –20°C for <1 week. To remove precipitated proteins, samples were centrifuged at ~15,000 x g for 10 minutes. A fraction (200 µl) of the supernatant was used for the HPLC analysis of low molecular weight thiols while the pellet was stored at –20°C for < 1 week for subsequent protein assay.

Anion Exchange HPLC Analysis of Low Molecular Weight Thiols. Low molecular weight thiols were analyzed as described by Fariss and Reed (23) with some modifications. Briefly, iodoacetic acid (IAA) was added to the 200 µl sample containing 10% perchloric acid to a final concentration of 130 mM and the pH was raised to ~8.5 by addition of dry potassium bicarbonate. An equal volume of fluorodinitrobenzene (FDNB) in 100% ethanol was added to a final concentration of 0.5% and incubated overnight at 4°C. Samples were separated on an aminopropyl anion exchange column. Compounds were identified by coelution with authentic pure compounds.

Specific activity of cellular glutathione. Cells labeled with Tran^{35}S-label (ICN Pharmaceuticals, Inc., Costa Mesa, CA) were lysed and treated as above for analysis of low molecular weight thiols. Samples were prepared for anion exchange HPLC analysis of low molecular weight thiols as described. The GSH and GSSG peaks were collected and counted using a Beckman LS-100C scintillation counter.

Assay of S-thiolated H-Ras. S-thiolated H-Ras was determined using a modification of the procedure of Fariss and Reed (23). Briefly, H-Ras was precipitated by adding 70% perchloric acid to a final concentration of 10%. After centrifugation at 15,000 x g, the supernatant was removed and used for quantitation of GSSG/GSH by HPLC. The pellet was washed with ice-cold 100% ethanol. The pellet was then resuspended in 50 mM MOPS pH 8.0 ± 50 mM DTT and incubated for 60 minutes at 37°C. Perchloric acid was again added to a final concentration of 10% and the sample was centrifuged at 15,000 x g. The supernatant was recovered and thiols were analyzed by anion exchange HPLC as described above.

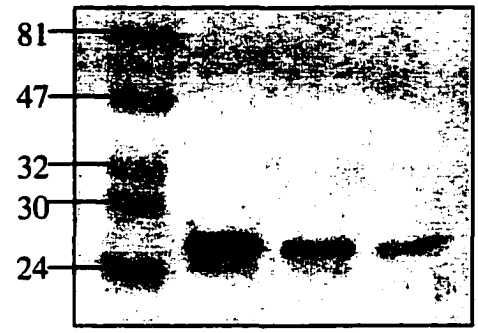
Results

Characterization of H-Ras. Separation of purified H-Ras by SDS-PAGE revealed only a single band (Figure 1A). The protein appears to be >90% pure as assessed by the dilution series. Isoelectric focusing (IEF) can be used to separate soluble proteins differing in charge. Since S-glutathiolation adds a single negative charge to proteins, the modified forms migrate at a more acidic pI than the unmodified proteins. Figure 1B shows the effect of addition of either uncharged iodoacetamide (IAM), or negatively charged iodoacetic acid (IAA) on H-Ras. The IAM-treated lane (left lane) shows two bands that have been previously reported to have isoelectric points (pIs) of 4.3 and 4.1 (21). The band that migrates at 4.3 is probably the reduced form of the protein (21), while the band at 4.1 is probably the protein with a single oxidized cysteine residue. Approximately 20% of the pure, unmodified protein was present as a more acidic band. The IAA-treated protein (right lane) focuses in two acidic bands with pIs of 4.0 and 3.9. These bands are thought to result from 2 and 3 modifications with the negatively charged reagent. Approximately 90% of the

Figure 1. Characterization of H-Ras preparation.

A. H-Ras was separated by SDS-PAGE as described in Materials and Methods. B. H-ras (10 μM) was reacted with 10 mM DTT for 30 minutes at pH 8.0 and 37°C. The reaction mixture was diluted by one-half and H-Ras was alkylated with either 20 mM IAM in 20 mM Tris-Cl buffer (pH 8.0) or 40 mM IAA in 60 mM Tris base (final pH = 7.5) and room temperature in the dark. The protein (1 μg) was then separated by IEF as described in Materials and Methods. C. IEF/Western blot of purified H-Ras and extracts of E. coli which overexpress wildtype and mutant forms of H-Ras treated with IAM and IAA. E. coli overexpressing H-Ras were cultured and lysed as described in Materials and Methods. Extracts and purified H-Ras were then treated with either 40 mM IAM or 40 mM IAA at pH 7.5 for 15 minutes. Extracts and purified H-Ras were then separated and visualized by IEF/Western blot analysis as described in Materials and Methods.

A.


81
47
32
30
24
kDa
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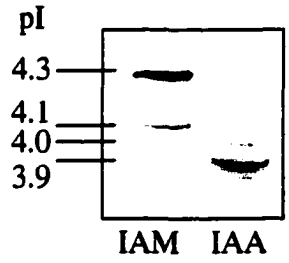
3 µg
1 µg
0.3 µg

B.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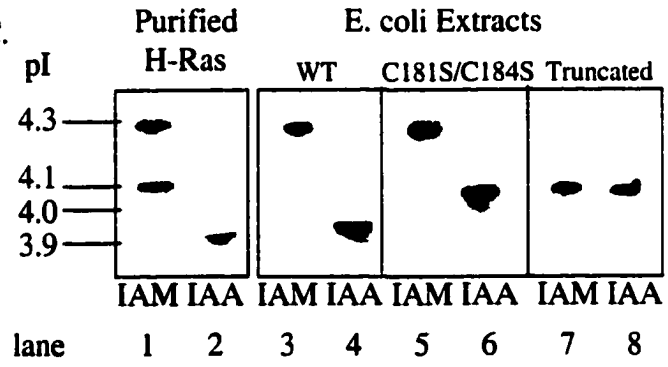

Purified H-Ras
E. coli Extracts
WT
C181S/C184S
Truncated
pI
4.3
4.1
4.0
3.9
IAM IAA IAM IAA IAM IAA IAM IAA
lane 1 2 3 4 5 6 7 8

IAA-treated H-Ras has 3 additional negative charges while 10% has two negative charges added or 2.9 moles adducts /mole of H-Ras. IAA, therefore, readily reacts with at least 3 cysteines in native H-Ras. Figure 1C shows IEF separations coupled with western blot analysis of either purified H-Ras or *E. coli* extracts containing wildtype or mutant H-Ras. Purified H-Ras (lanes 1,2) and wildtype extracts (lanes 3,4) show mobilities identical to each other after IAM or IAA treatment. H-Ras in which the cysteines at positions 181 and 184 are mutated to serine (C181S/C184S, lanes 5,6) shows IAA reactivity with only one cysteine. The truncated form of H-Ras, which does not have the three c-terminal cysteine residues, does not react with IAA (lanes 7,8). This experiment therefore identifies the three IAA-reactive cysteines as cysteines 181, 184, and 186. Cysteine 118, which is surface exposed in X-ray crystal structures and NMR structures of H-Ras (8,27), does not appear to be reactive towards IAA.

Reactions of H-Ras with disulfides. The reaction of a protein with GSSG to form the S-glutathiolated protein, while probably of little physiologically importance (28, see chapter I), demonstrates the potential reactivity of H-Ras cysteines to S-glutathiolation (29). Figure 2 shows the reaction of H-Ras under conditions that enhance the rates of thiol-disulfide exchange, i.e. pH 8.0, 20 mM GSSG. Lanes 1 and 2 are control lanes that identify the multiple charge forms of H-Ras before and after modification. Lanes 4 through 6 show a time course of the reaction of H-Ras with GSSG. After 1 minute reaction with GSSG (lane 4), the reduced band (pI = 4.3) disappears and H-Ras migrates as two bands pIs of 4.1 and 4.0. These bands apparently result from the complete modification of one cysteine and the partial modification of a second. By one hour (lane 6) at least two cysteines are completely modified and bands for modification of all four reactive cysteines are visible. If H-Ras is

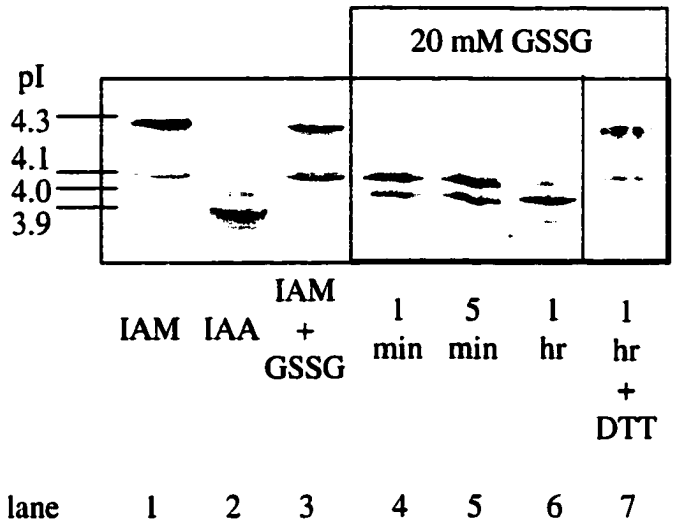


Figure 2. Reaction of H-Ras with GSSG.

H-Ras (10 μM) was incubated with 20 mM GSSG for the indicated times at 37°C and pH 8.0. The reactions were stopped by alkylation with IAM. The alkylation and reduction of the protein were performed as described in Figure 1B. Lane 3 shows treatment with 10 mM IAM at pH 8.0 for 5 minutes before GSSG addition.

incubated with dithiothreitol after reaction with GSSG (lane 7), it migrates as the untreated protein. This shows that the acidic bands are reductant-labile, another characteristic of S-glutathiolated protein. If the protein is incubated with iodoacetamide previous to GSSG addition (lane 3), the reaction is blocked.

If H-Ras is modified with a positively charged adduct, the modified protein should focus with a more basic pI. Figure 3 shows that when H-Ras is incubated with the positively charged disulfide, cystamine (lanes 2,3), it migrates at a more basic pI, marked "2" in the gel. The presence of a faint band at the position marked "1" suggests that there is one intermediate site in this reaction. The modified H-Ras can be returned to its original state by reduction with DTT (lane 4). This shows that H-Ras can be S-cystamylated on at least 2 cysteine residues.

H-Ras reactions with Hydrogen Peroxide. Since S-glutathiolation in cells probably occurs by direct oxidation of protein sulfhydryls (28), the reaction of H-Ras with H_2O_2 in the presence of 3 mM GSH was studied (Figure 4A). H-Ras was very rapidly modified on one site by H_2O_2 (lane 3) and this modification was DTT-reducible (lane 4). It is likely that this modification was S-glutathiolation. Lanes 5 and 6 show that 1.5 mM GSSG, the most which could possibly form in the reaction of 3.0 mM GSH and 2.0 mM H_2O_2, did not modify H-Ras to the extent seen in the H_2O_2 + GSH reaction. Thus, GSSG was not an intermediate in S-glutathiolation of H-Ras in the H_2O_2 + GSH reaction. GSH was released from H-Ras and identified by coelution with GSH standards using anion exchange HPLC. S-glutathiolation calculated using HPLC (Figure 4B) is almost identical to the amount of S-glutathiolation as calculated from the IEF gel (Figure 4A, lanes 3,4). Concentration of GSSG in this same reaction mixture was approximately 52 μM. This means that 35% of the original GSH was

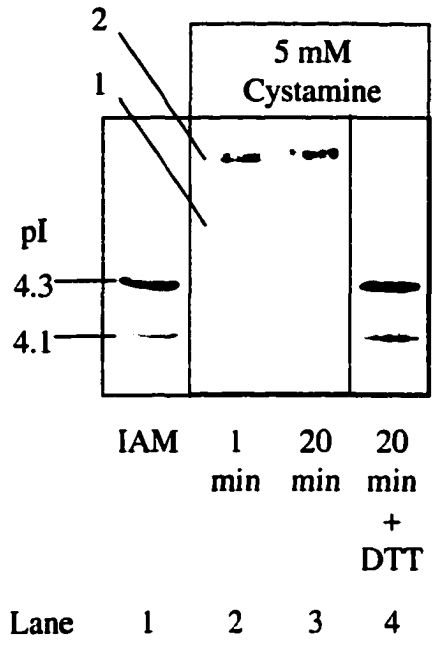


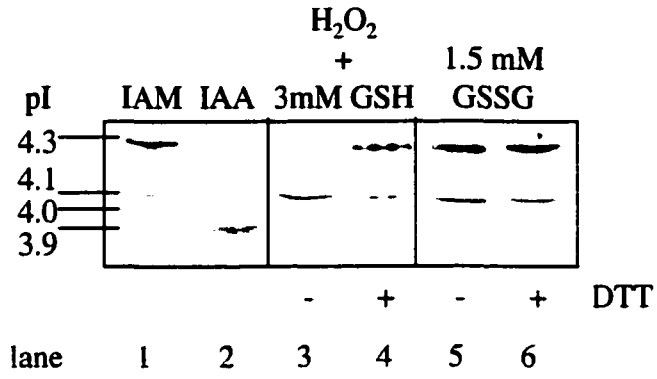
Figure 3. Reaction of H-Ras with Cystamine.

H-Ras (10 μM) was incubated with 5 mM cystamine for the indicated times at 37°C and pH 8.0. The reactions were stopped by alkylation with IAM. The alkylation and reduction of the protein were performed as described in Figure 1B.

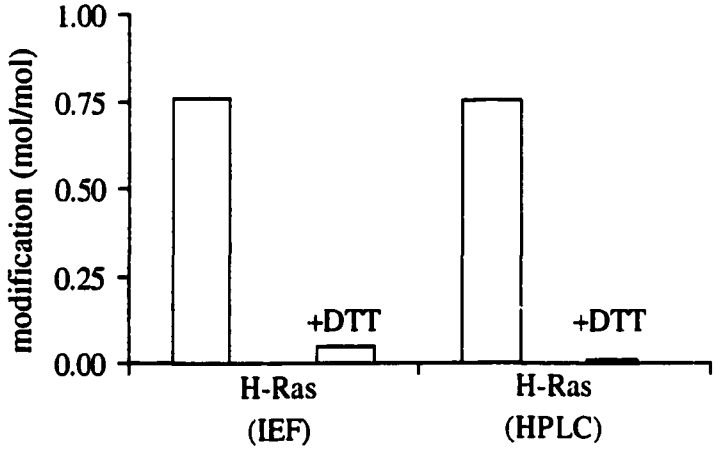
Figure 4. Reaction of H-Ras with Hydrogen Peroxide.

A. H-Ras (10 μM) was incubated with 2 mM H_2O_2 + 3 mM GSH (lanes 3,4) or 1.5 mM GSSG (lanes 5,6) for 5 minutes at 37°C and pH 8.0. The reactions were stopped by alkylation with IAM. The alkylation and reduction of the protein were performed as described in Figure 1B. B. An aliquot of H-Ras, incubated with H_2O_2 + GSH (±DTT) from part A (lanes 3,4), was assayed for release of GSH as described in Materials and Methods. The graph shows fractional modification (mole/mole) of H-Ras as calculated from IEF (described in Materials and Methods) and as determined by HPLC glutathione analysis. C. H-Ras (10 μM) was incubated with 2 mM H_2O_2 ± 0.3 mM GSH at 37°C and pH 8.0. The reactions were stopped by alkylation with IAM. The alkylation and reduction of the protein were performed as described in Figure 1B. IEF separation was performed as described in Materials and Methods. Modification was determined by quantifying bands of the IEF separation. Reversible modification is the difference in modification between DTT-untreated and DTT-treated lanes. Irreversible modification is modification in DTT-treated lanes minus the modification of untreated H-Ras protein.

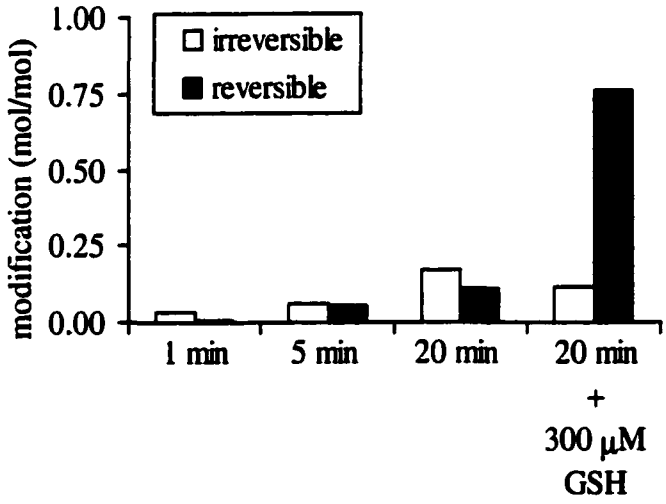
A.


H_2O_2
+
3mM GSH
1.5 mM
GSSG
pI
IAM
IAA
4.3
4.1
4.0
3.9
-
+
-
+
DTT
lane
1
2
3
4
5
6

B.


modification (mol/mol)
1.00
0.75
0.50
0.25
0.00
+DTT
+DTT
H-Ras
(IEF)
H-Ras
(HPLC)

C.


modification (mol/mol)
1.00
0.75
0.50
0.25
0.00
irreversible
reversible
1 min
5 min
20 min
20 min
+
300 μM
GSH

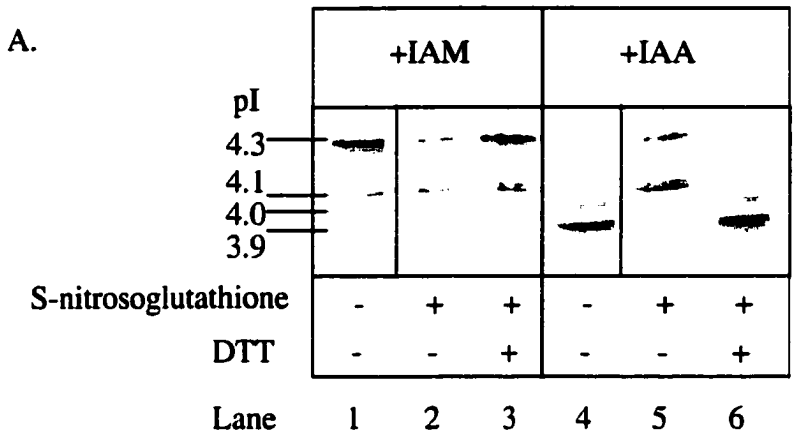
oxidized to form GSSG. This suggests that the reactivity of one H-Ras cysteine is better than free GSH in this reaction. Figure 4C shows calculated irreversible and reversible modification of H-Ras in a reaction with H_2O_2. In the absence of GSH, less than 20% of one thiol is irreversibly oxidized even after 20 minutes, but as little as 0.3 mM GSH is necessary to cause S-glutathiolation of H-Ras on 75% of the protein (Figure 4C). Irreversible oxidation of H-Ras by H_2O_2 is not as rapid as S-glutathiolation. This has also been observed in studies of carbonic anhydrase III (see Chapter I).

H-Ras reaction with S-nitrosoglutathione. S-nitrosylation has been increasingly recognized as an important oxidative modification of proteins (21,30-32). Pure H-Ras has been shown to react with NO on Cys118 in mutants that do not contain the c-terminal tail (truncated mutant) (6) and on at least three cysteines in extracts of *E. coli* which overexpress wildtype H-Ras (21). H-Ras may also react with NO *in vivo* as part of signal transduction processes (6). Figure 5A, lanes 1-3 show H-Ras incubated with IAM after S-nitrosoglutathione incubation. Lane 2 shows that some S-glutathiolation occurs, since the protein appears about equally distributed between the reduced (pI = 4.3) and singly modified isoforms (pI = 4.1). This modification was reversible with DTT treatment (lane 3). Because S-nitrosylation is an uncharged modification of protein cysteine, H-Ras was reacted with IAA after S-nitrosoglutathione incubation. S-nitrosylation causes the inhibition of IAA derivitization. When purified H-Ras was incubated with S-nitrosoglutathione, as many as three cysteines were S-nitrosylated, as indicated by inhibition of IAA reaction (lanes 4,5). S-nitrosylation is a reversible modification, so DTT treatment (lanes 6) reduced the S-nitrosylated H-Ras and restored the IAA-modified forms. When S-nitrosoglutathione was reacted with extracts of *E. coli* that overexpress C181S/C184S mutant H-Ras, nearly one full site was S-nitrosylated,

Figure 5. Reaction of H-Ras with S-nitrosoglutathione.

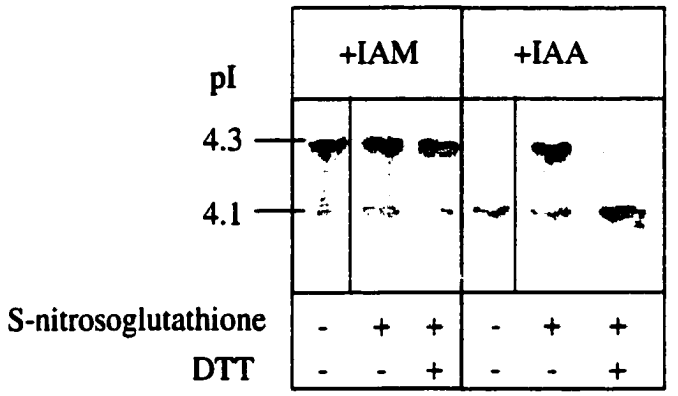
A. H-Ras(10 μM) was incubated with 5 mM S-nitrosoglutathione for 5 minutes at 37°C and pH 8.0. The reaction was stopped by alkylation with IAM (lanes 1,2) or IAA (lanes 4,5) as described in Figure 1B. The protein was reduced (lanes 3,6) as described in Figure 1B and alkylated with either IAM (lane 3) or IAA (lane 6). B. IEF/Western blot of extracts of E. coli that overexpress C181S/C184S mutant of H-Ras treated with S-nitrosoglutathione. *E. coli* overexpressing H-Ras were cultured and lysed as described in Materials and Methods. Extracts were then treated with 5 mM S-nitrosoglutathione for 15 minutes. The reaction was stopped with 25 mM IAM or 40 mM IAA at pH 7.5. DTT treatment was 10 mM for 15 minutes followed by reaction with IAM or IAA. Extracts were then separated and visualized by IEF/Western blot analysis as described in Materials and Methods. C. IEF/Western blot of extracts of *E. coli* that overexpress the truncated mutant of H-Ras treated with S-nitrosoglutathione. *E. coli* overexpressing H-Ras were cultured and lysed as described in Materials and Methods. Extracts were then treated with 5mM S-nitrosoglutathione for 15 minutes. The reaction was stopped with 20 mM Cystamine at pH 7.5. DTT treatment was 10 mM for 15 minutes followed by reaction with cystamine. Extracts were then separated and visualized by IEF/Western blot analysis as described in Materials and Methods.

A.



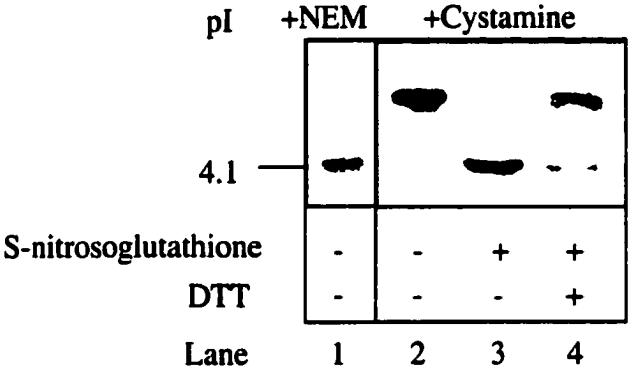
Purified H-Ras

B.



C181S/C184S

C.



Truncated Mutant

and again small amounts of S-glutathiolation occurred (Figure 5B). Cys118 was previously shown to be S-nitrosylated with authentic NO. However, IAA did not react with Cys118 (Figure 1), so it was necessary to determine whether Cys118 would be S-nitrosylated by S-nitrosoglutathione using the truncated mutant. Incubating extracts of E. *coli* that overexpress the truncated mutant H-Ras with cystamine (Figure 5C, lane 2) showed a shift in H-Ras migration to a more basic pI compared to NEM-treated H-Ras (lane 1). Thus, Cys118 was one of the sulfhydryls that reacted with cystamine. If the extract was incubated with S-nitrosoglutathione before cystamine treatment (lane 3), the cystamine reaction was inhibited. This inhibition was removed by DTT incubation (lane 4). Thus it was found that S-nitrosoglutathione reacts with H-Ras to form S-nitrosylated H-Ras on at least four cysteines and is S-glutathiolated on one cysteine.

These experiments illustrate that *in vitro*, the cysteine residues of H-Ras will participate in a wide variety of thiol oxidations including S-glutathiolation, S-nitrosylation, and irreversible oxidation.

S-thiolation of H-Ras in NIH-3T3 cells. The cysteines of H-Ras have individual functions *in vivo*. Lipids modify all three cysteines on the c-terminus of H-Ras; Cys186 is prenylated, and Cys181 and 184 are palmitoylated. The availability of these cysteine residues for oxidative modification *in vivo* is unknown. To address this question, NIH-3T3 cells overexpressing either wildtype (wt), C118S, or C181S/C184S H-Ras were oxidized with diamide. Diamide produces extensive and reversible S-thiolation *in vivo* (20,33). Figure 6 shows the effects of several concentrations of diamide on total protein S-thiolation of WT, C118S, and C181S/C184S expressing cells. In all 3 cell types 2 mM diamide produces

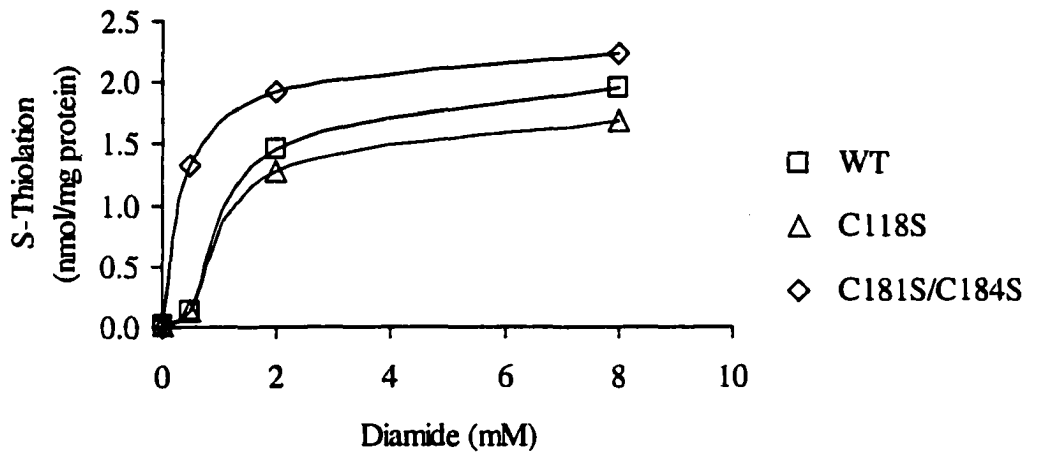


Figure 6. S-thiolation of total cytosolic proteins in cells overexpressing mutant forms of H-Ras by the thiol oxidant diamide.

NIH-3T3 cells overexpressing wildtype and mutant forms of H-Ras were labeled with Tran^{35}S-label for 4 hours to achieve a specific activity of glutathione of approximately $5x10^7$ cpm/μmol. Labeling was carried out in the presence of cycloheximide (100 μg/ml) to prevent protein synthesis during glutathione labeling (38). Cells were treated with diamide for 10 minutes, lysed with 20 mM β–glycerophosphate buffer containing 50 mM NEM, spun at 160,00 x g to remove insoluble proteins, and spotted on filter paper for processing. Filter squares were washed in 10% TCA to remove non-protein counts as described previously (38). Control samples were treated with 50 mM DTT for 30 minutes at 37°C before spotting on paper to determine background incorporation into proteins. S-thiolation is measured in nmols of GSH incorporated into proteins per mg total protein.

maximal S-thiolation, approximately 5 nmols adduct/mg of total cellular protein.

Figure 7 shows an IEF gel of the reaction of pure H-Ras with diamide in the presence of GSH *in vitro*. Lane 3 shows that the reacted protein migrates at a pI indicating addition of two glutathione adducts (pI = 4.0). This modification is reversible with DTT (lane 4). This demonstrates that diamide will effectively cause S-glutathiolation of at least two sites on H-Ras *in vitro*.

To examine the extent of H-Ras S-thiolation in cells expressing various H-Ras proteins, H-Ras was immunoprecipitated from cells labeled with ^{35}S-cysteine. Figure 8 shows an SDS-PAGE gel of H-Ras from cells overexpressing wildtype (lanes 1,2), C118S (lanes 3,4), or C181S/C184S (lanes 5,6) H-Ras. The top of the figure shows the Coomassie blue stained bands of the immunoprecipitated H-Ras and the bottom shows the autoradiograph of the radioactivity associated with the bands. Reduction sensitive radioactivity was associated with the H-Ras band from each of these cells (compare lanes 1, 3, and 5 with lanes 2, 4, and 6, respectively), indicating that H-Ras was S-thiolated under these conditions. WT H-Ras was not S-thiolated in untreated cells (not shown). This experiment demonstrates the potential for oxidative modification of H-Ras in cells. Since all three forms of the protein were glutathiolated, neither Cys118, Cys181 nor Cys184 is necessary for S-glutathiolation of the protein. Cys186 may be the primary site of S-thiolation *in vivo*.

Discussion

From this data it is possible to propose the model shown in Figure 9. Based on mutational analysis, H-Ras reacts with negatively charged IAA on three sites, Cys181, Cys184, and Cys186. Of the six cysteine residues on H-Ras, four, cysteines 118, 181, 184,

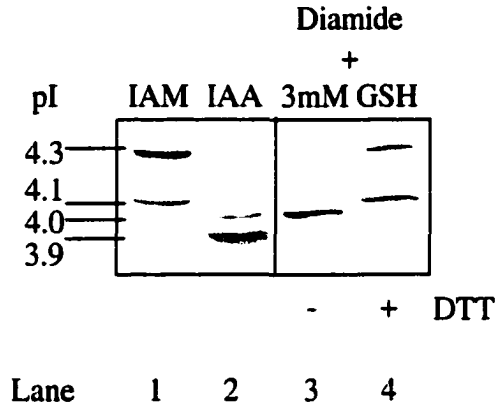
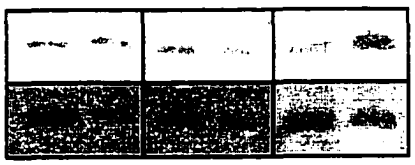


Figure 7. Reaction of H-Ras with Diamide.

H-Ras (10 μM) was incubated with 2 mM diamide + 3 mM GSH for 5 minutes at 37°C and pH 8.0(lanes 3,4). The reaction was stopped with IAM (lane3) or by reduction with DTT and then alkylation with IAM (lane 4). Alkylations and reduction were performed as described in Figure 1B.

Figure 8. S-thiolation of wildtype and cysteine mutants of H-Ras in NIH-3T3 cells treated with diamide.

Cells overexpressing WT, C118S, or C181S/C184S H-Ras were labeled with Tran^{35}S-label for 4 hours in the presence of cycloheximide (100 μg/ml), then treated with 2 mM diamide for 10 minutes. Cells were then washed twice with ice cold PBS and lysed with immunoprecipitation buffer (TBS/1% triton x-100/0.5% Sodium Deoxycholate/0.1% SDS) containing 50 mM NEM to stop artifactual modification of proteins. Lysates were centrifuged at 15,000 x g and H-Ras was immunoprecipitated from cellular lysates with agarose-conjugated Ab-1. The supernatant was then incubated at 4°C with the immunoprecipitation beads for 2 hours. Then the immunoprecipitation beads were washed twice in immunoprecipitation buffer and layered over immunoprecipitation buffer + 7.5% sucrose twice. Approximately 5 μl of beads were used per 0.5 mg total protein to yield 0.1-0.2 μg of H-Ras. H-Ras was released from the immunoprecipitation beads by boiling for 5 minutes in SDS-PAGE sample buffer containing 10 mM NEM. Replicate lanes were treated with 10 mM DTT before NEM treatment to release reduction-sensitive radioactivity. Gels were stained with Coomassie blue, dried and exposed to film. Both radioactive and Coomassie blue-stained bands were quantified using densitometry to determine radioactivity relative to the protein stain. Ratios are normalized to the background incorporation after DTT treatment.

	WT		C118S		C181S/ C184S	
Coomassie Blue / Autoradiogram						
DTT	-	+	-	+	-	+
Autoradiogram Band Density	5.2	1.0	2.3	1.0	4.4	1.0
Lane	1	2	3	4	5	6

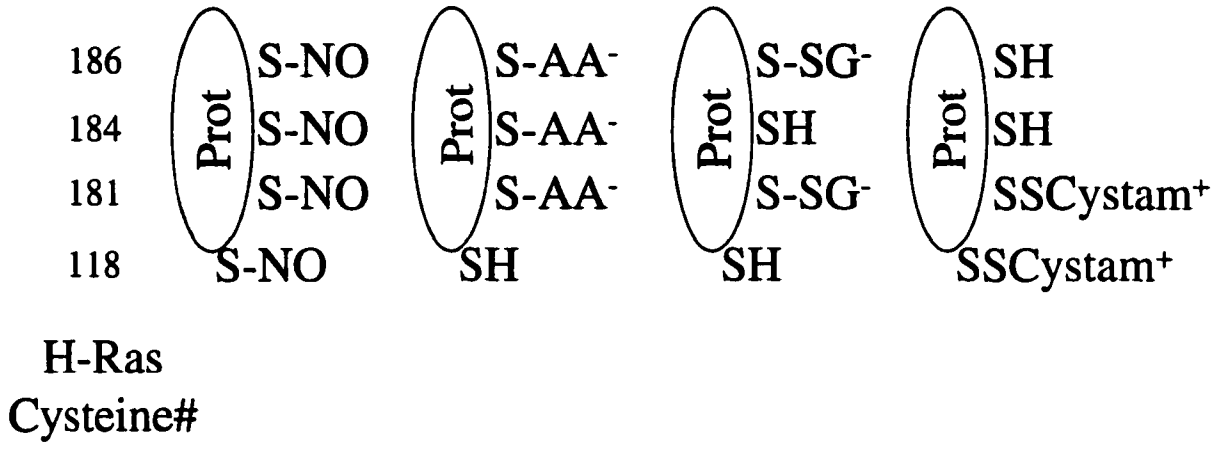


Figure 9. Model for reactivity of sulfhydryls of H-Ras.

The S-nitrosylation (S-NO) and modification of cysteines by IAA ($S\text{-}AA^-$) were positively identified by mutational analysis of reactivity. S-cystamylation of Cys118 ($SSCystam^+$) was identified by mutational analysis of reactivity. S-cystamylation of Cys181 was inferred by reactivity of cystamine with up to two sites and by analysis of the H-Ras sequence; reactivity with Cys184 or Cys186 is also possible. S-glutathiolation of Cys181 and 186 ($S\text{-}SG^-$) was inferred by reactivity with two sites, reactivity with IAA, electrostatic analysis, and analysis of the H-Ras sequence. Trace S-glutathiolation on two additional sites was observed in IEF gels.

and 186, are likely to be surface exposed as shown by structural, chemical, and mutational studies (8,10,27). Figure 10A shows an electrostatic surface calculation of the area on H-Ras surrounding Cys118. While the sulfur atom appears to be surface accessible, it resides within a region of negative charge. On this basis, Cys118 should not react with negatively charged IAA.

Positively charged cystamine does react with Cys118. It is likely that the negative charge environment around Cys118 enhances this reaction. While the structure of the c-terminal cysteines is not available for analysis, the sequence is available (Figure 9B). It is reasonable to suggest that lys185, which carries a positive charge at physiological pH, can affect the charge environment of cysteines 184 and 186 enough to inhibit reactions with positively charged reagents. Thus, it is most likely that Cys181 is the second cysteine that reacts with cystamine.

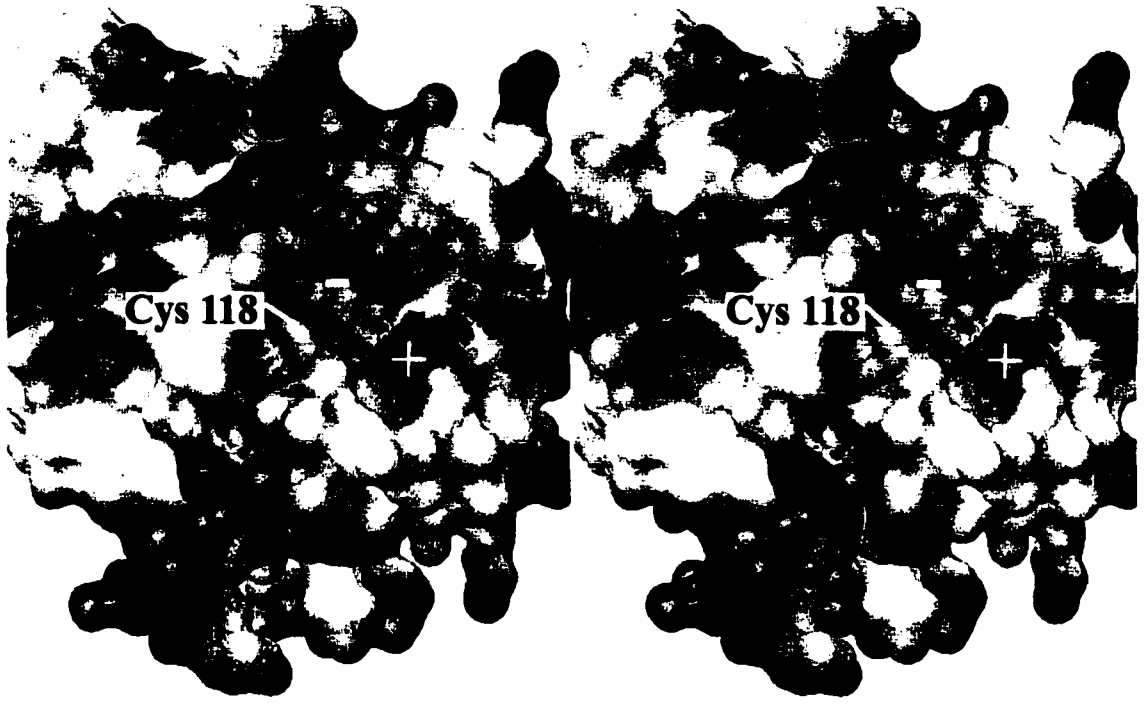
H-Ras can be readily S-glutathiolated on at least two sites. IAA, which also carries a negative charge, reacts with three cysteine residues. Since IAA does not react with Cys118, it is likely that GSH will also not react at that site. It is therefore probable that the c-terminal cysteines are S-glutathiolated. Lys185 may enhance reactivity of Cys184 and Cys186, however, steric interference may prevent simultaneous formation of adducts at both Cys184 and 186. This may explain the differences in reactivity of IAA and S-glutathiolating agents. Therefore Cys181 and either Cys184 or Cys186 are likely the two residues which are S-glutathiolated, based on the reactivity of similarly charged IAA, the electrostatic surface map, and the steric considerations discussed above.

In contrast to S-glutathiolation, the S-nitrosylating agent S-nitrosoglutathione readily modified all four available cysteine residues *in vitro* (Figure 5). This data suggests that

Figure 10. Environment of H-Ras Cysteine residues.

A. A stereo diagram shows an electrostatic surface calculation of the area surrounding Cys118. The X-ray crystal structure of H-Ras (PDB Accession code 121P) was used in electrostatic surface charge calculations with MolMol (34). The sulfur atom of Cys118 is labeled. Positively charged areas are black, neutral regions are white and negatively charged regions are grey. The positive region nearest Cys118 is marked with a "+" while the nearest negative region is highlighted with a "-". B. The sequence of amino acids surrounding Cys181, 184, and 186. The sequence was obtained from GenBank (#NM_005343).

A.



B.

S-nitrosylation is a very non-specific reaction, dependent perhaps only on the nucleophilic character and surface exposure of the thiol in question. Further experiments may clarify this question in intact cells.

The *in vivo* data (Figure 8) suggests that while cysteines 118, 181, and 184 may be S-glutathiolated in cells, none of them are required for S-thiolation. This agrees with *in vitro* data that diamide and GSH modify multiple thiols (Figure 7). Because cycloheximide treatment and the overexpression of different mutants of H-ras may alter membrane association of H-Ras (11), and thus, availability of cysteines 181, 184 and 186, direct comparisons of relative reactivity may not be possible between the different mutants.

We can hypothesize that specificity for a particular adduct on a particular site can be achieved through the charge environment of that site. An uncharged adduct such as NO would be equally likely to modify positively and negatively charged sites, while a negatively charged adduct like glutathione would preferentially react with positively charged sites. Because many cysteine sulfurs are thought to create a negative charge environment (35), a site with a positive charge would potentially be highly favored *in vivo* when competing with protein sulfhydryls for available GSH. The charge environment may be changed significantly by the addition of glutathione, providing a potential mechanism for allosteric effects.

In reactions of cysteines with H_2O_2, the formation of a charged adduct such as the sulfinic acid is a two-step reaction, with sulfenic acid as the intermediate. In the presence of GSH, the predominant form of oxidized cysteine is the S-glutathiolated cysteine. This provides the obvious advantage over irreversible oxidation in that the S-glutathiolated cysteine is reducible by thiol-disulfide exchange. This means that regulation by this

mechanism can be reversible (36,37). It is also possible that the size and charge characteristics of the glutathione might allow recognition by a protein in addition to possible allosteric changes associated with the adduct.

Because negatively charged IAA does not react with Cys118, this site is probably not S-glutathiolated efficiently, and regulation at this site by S-glutathiolation is not likely in H-Ras. S-nitrosylation of this site *in vitro* has been confirmed (6,21) and this site may still be regulated by S-nitrosylation *in vivo*. However, the oxidation of the c-terminal cysteine residues is likely *in vivo*, which means that lipidation may be regulated by oxidation *in vivo* through S-glutathiolation in cells under oxidative stress, or through S-nitrosylation in cells under nitrosative stress. Whether this regulation would be occur by direct inhibition of lipidation or through a more complex mechanism is uncertain. If the lipidation sites and Cys118 are modified oxidatively or nitrosatively *in vivo*, then regulation through these mechanisms could be concentration and/or time dependent. The total activity of H-Ras would be the sum total of the negative regulation by inhibition of lipidation (10,11) and the positive regulation of Cys118 allosteric effects (6).

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CHAPTER III:

S-NITROSYLATION AND S-GLUTATHIOLATION OF H-RAS *IN VIVO* BY S-NITROSOCYSTEINE

A paper to be submitted to Archives of Biochemistry and Biophysics

Robert J. Mallis and James A. Thomas

Abstract

The effect of S-nitrosocysteine on cells has been attributed primarily to protein S-nitrosylation. In this report the effect of S-nitrosocysteine on protein S-nitrosylation and S-glutathiolation of the low molecular weight G-protein H-Ras in NIH-3T3 cells is studied. S-nitrosocysteine caused the formation of S-nitrosoglutathione, cysteine-glutathione disulfide, cysteine, cystine, and glutathione disulfide in NIH/3T3 cells. Additionally, it was found that S-nitrosocysteine caused S-nitrosylation, S-glutathiolation and S-cysteylation of the cytosolic protein pool. S-nitrosylation was the dominant modification, but S-glutathiolation and S-cysteylation accounted for approximately10-20% of the total protein thiol modification. H-Ras, immunoprecipitated from NIH-3T3 cells overexpressing wildtype H-Ras (NIH-3T3/WT) and treated with 1 mM S-nitrosocysteine, was found to contain approximately 0.85 moles S-nitrosocysteine per mole of H-Ras protein as determined by nitrite release from the protein. In a similar experiment, H-Ras was immunoprecipitated from NIH-3T3/WT cells in

which the glutathione pool was radiolabeled with ^{35}S-cysteine. S-nitrosocysteine (1 mM) caused formation of approximately 0.16 moles S-glutathiolated protein cysteine per mole H-Ras as determined by release of protein bound radioactivity by dithiothreitol. These results suggest that both S-nitrosylation and S-glutathiolation can regulate H-Ras. These results also suggest that S-nitrosocysteine enters cells intact, modifying both protein and low molecular weight thiols by a variety of reactions. S-nitrosylation may be an important but not unique modification of H-Ras that results from an extracellular S-nitrosocysteine pool.

Introduction

H-Ras, a low molecular weight G-protein, is an integral component of the extracellular signal-regulated kinase (Erk)[1] pathway (1). H-Ras is necessary for the activation of this pathway by reactive oxygen species such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) (2-5). Pure H-Ras can be S-nitrosylated on as many as four cysteine residues (see Chapter II) *in vitro* by the NO-donating compound S-nitrosoglutathione or on at least one cysteine by NO (7). This data has led to speculation that S-nitrosylation of H-Ras can be a mechanism for activation the Erk pathway *in vivo* (7). Our recent work has also shown that H-Ras can be S-thiolated, i.e. oxidized to form a protein-thiol mixed disulfide, *in vitro* and *in vivo* by thiol oxidants including H_2O_2, diamide, and glutathione disulfide

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[1] Abbreviations used in this paper: DTT, dithiothreitol; Erk, extracellular signal-regulated kinase; GSH, reduced glutathione; GSSG, glutathione disulfide; IAA, iodoacetic acid; IAM, iodoacetamide; IEF, isoelectric focusing; NEM, N-ethylmaleimide.

(GSSG)[2] (see Chapter II). This raises the possibility that the Erk pathway can also be regulated by S-thiolation of critical cysteine residues of H-Ras.

NO and its related compounds are thought to regulate diverse processes *in vivo*, including vasodilation (8), platelet aggregation (9), apoptosis (10,11), and cell proliferation (7). Although NO regulates vasodilation by stimulating guanylate cyclase to form cyclic guanosine monophosphate (cGMP) (8), the mechanisms for regulation of other processes are less well understood. A class of NO-related compounds that may be responsible for some of its biological activity is the S-nitrosothiol. The mechanism of synthesis of S-nitrosothiols *in vivo* is the subject of considerable research (12-16). S-nitrosothiols have been found in low micromolar concentrations in various tissues and the possibility for higher concentrations exists in situations such as induction of nitric oxide synthase enzymes either within cells or during the respiratory burst of immune cells (17). S-nitrosothiols may be NO-carriers in the induction of vasodilation (17,18) and may also be integral in regulation of platelet aggregation (9). NO regulation of many processes may be mediated by the formation of S-nitrosothiols on critical protein sites. S-nitrosylation of a protein can affect the activity of that protein either through direct inhibition of the active site, as shown *in vitro* with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (19) and caspase (10,11), or through allosteric effects, as is suggested with H-Ras (7).

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[2] A note on nomenclature: GSH refers ONLY to the reduced form of glutathione. GSSG refers only to glutathione disulfide. Oxidized glutathione may refer to several forms of glutathione including GSSG, cysteine-glutathione disulfide, S-glutathiolated protein and S-nitrosoglutathione.

The addition of S-nitrosothiols to mixtures of thiols *in vitro* initiates two main phenomena. First, NO^+ is transferred very rapidly from the S-nitrosothiol to free thiols until thermodynamic equilibrium is achieved (20). If thiols are in excess of the S-nitrosothiols, then near quantitative transfer of the NO^+ to the thiols will be achieved (20). This applies to both protein thiols and low molecular weight thiols, including reduced glutathione (GSH) and cysteine (20,21). The second phenomenon that occurs when S-nitrosothiols are mixed with thiols is the degradation of the S-nitrosothiol to its disulfide and nitrite over time. This multistep process is accelerated by the presence of free thiols (21,22), is accelerated by oxygen (22), and occurs more slowly than transnitrosation reactions (20,21,22).

In cells, the fate of S-nitrosothiols is uncertain. GSH and protein cysteine concentrations are very high in cells. At low concentrations of S-nitrosothiol, transfer of NO^+ to endogenous thiols may be nearly quantitative if breakdown of the S-nitrosothiol does not occur by other mechanisms first (20,22). The consequences of this would be S-nitrosylation of GSH and protein cysteines. Breakdown of an S-nitrosothiol before it enters the cells, or breakdown of S-nitrosothiols within the cell might produce a variety of reaction products (22). These possible products include disulfides, including protein-glutathione mixed disulfides (S-glutathiolated proteins) as well as disulfides of the original NO^+ donor, sulfinic and sulfonic acids, nitrite, nitrous oxide, and ammonia (22,23). Additionally, interactions with other reactive oxygen species such as superoxide might lead to still more complex reactions (24,25). These studies suggest that S-nitrosylation of proteins is a likely outcome of addition of S-nitrosothiols to cells, however, other oxidations including S-glutathiolation of proteins are possible. S-glutathiolation of a protein cysteine residue could have effects similar to S-nitrosylation if formation of an adduct inhibits chemical reactivity

of that cysteine. However, if allosteric effects or effects on protein-protein interactions are thought to occur, then S-nitrosylation and S-glutathiolation might have different consequences.

In this paper, we study the reactions between S-nitrosocysteine and endogenous thiols in NIH-3T3 cells. The effects of S-nitrosocysteine on low molecular weight thiols, total protein thiols and H-Ras were determined. Data presented here shows that S-nitrosocysteine causes significant oxidation and S-nitrosylation of the low molecular weight thiols as well as S-nitrosylation, S-glutathiolation, and S-cysteylation of total protein thiols. H-Ras is found to be both S-nitrosylated and S-glutathiolated under these conditions.

Materials and Methods

Materials. l-cysteine, d-cysteine, Dithiothreitol (DTT), reduced glutathione (GSH), glutathione disulfide (GSSG), iodoacetic acid (IAA), iodoacetamide (IAM), N-acetylpenicillamine, N-ethylmaleimide (NEM), and rat anti-mouse alkaline phosphatase-conjugated antibody were from Sigma, Inc (St. Louis, MO). Cell culture reagents were purchased from GIBCO-BRL (Gaithersburg, MD), with the exception of bovine calf serum (BCS) which was from HyClone (Logan, UT). Ampholytes were purchased from Amersham-Pharmacia (Piscataway, NJ). Agarose conjugated Ab-1 and recombinant human H-Ras were purchased from Calbiochem-Novabiochem (San Diego, CA). Monoclonal Ab 146-03E4 (Ab 146) was purchased from Quality Biotech (Camden, NJ). 2,3-diaminonaphthalene (DAN) was purchased from ICN Pharmaceuticals, Inc (Costa Mesa, CA).

Cell Culture. NIH-3T3 fibroblasts were obtained from American Type Culture Collection (ATCC) and cultured according to their recommendations (Dulbecco's Modified Eagle Medium (DMEM) + 2mM Glutamine + 0.2mM Sodium Pyruvate + 10% BCS + 100 units/ml each of Penicillin and Streptomycin). NIH-3T3 cells overexpressing wildtype H-Ras were a generous gift from L.A. Quilliam (Indiana University School of Medicine, Indianapolis, IN) and were cultured according to ATCC recommendations for NIH-3T3 cells.

Preparation of S-nitrosothiols. S-nitrosocysteine and S-nitrosoglutathione were prepared as previously described (6). Briefly, 220 µl each of 220 mM GSH and 220 mM sodium nitrite were mixed with 25 µl of 4.0 N HCl and incubated in the dark at room temperature for 10 minutes. The solution was then neutralized with 25 µl of 4.0 N NaOH to give a final concentration of approximately 100 mM S-nitrosoglutathione. The final concentration was calculated from absorbance at 334 nm using the extinction coefficient 767 M^{-1} cm^{-1} (26). S-nitroso-N-acetylpenicillamine was prepared as described (27). Briefly, 50 mg N-acetylpenicillamine was dissolved in 600 µl methanol + 120 µl 1N NaOH on ice. Sodium Nitrite (155 mg) was dissolved in 100 µl doubly distilled H_2O and added to the first solution. This solution was then acidified with 360 ml concentrated HCl to a pH of 1. The solution was then vortexed and left on ice. The resulting green solid precipitate was centrifuged at 15,000 x g and the pellet was washed three times with ice cold doubly distilled H_2O and dried in a vacuum. The resulting green solid was stored at -20°C. The dried solid was dissolved in PBS + 0.04 N NaOH and neutralized using 40 ml of 1 N HCl before addition to cultures. The concentration of S-nitroso-N-acetylpenicillamine was calculated using the absorbance at 338 nm and the extinction coefficient 890 $M^{-1}cm^{-1}$ (28).

Cellular experiments. NIH-3T3 cells were grown to confluence (assessed visually, approximately 1-2 x 10^6 cells/35 mm plate) and the medium was changed. Experiments were started 24 hours later. Experiments were conducted in phosphate buffered saline (PBS) to avoid any oxidative artifacts associated with interactions between DMEM and added oxidants. PBS was added to cells 5 minutes prior to the addition of oxidant to avoid oxidative artifacts associated with change of medium.

Anion Exchange HPLC Analysis of Low Molecular Weight Thiols. Low molecular weight thiols were analyzed as described by Fariss and Reed (29) with some modifications. Briefly, 20 µl of 1.3 M iodoacetic acid (IAA) was added to the 200 µl sample to a final concentration of 120 mM and the pH was raised to ~8.5 by addition of dry potassium bicarbonate. An equal volume of fluorodinitrobenzene (FDNB) in 100% ethanol was added to a final concentration of 0.5% and incubated overnight at 4°C. Samples were separated on an aminopropyl anion exchange column (CEL Associates, Inc. Houston, TX). All thiol compounds were identified by coelution with standards. S-nitrosoglutathione and disulfides were also identified by susceptibility to reduction with dithiothreitol (DTT). All compounds were previously identified with this methodology (6,29).

Analysis of Cellular Low Molecular weight thiols. NIH-3T3 cells were rinsed twice with ice cold PBS and lysed with 10% perchloric acid. Plates were scraped and the soluble and precipitated materials were collected and stored at –20°C for <1 week. To remove precipitated proteins, samples were centrifuged at ~15,000 x g for 10 minutes. A fraction (200 µl) of the supernatant was used for the HPLC analysis of low molecular weight thiols while the pellet was stored at –20°C for < 1 week for subsequent protein assay.

Protein assay. Protein concentration was determined as described by Lowry et al (30).

S-nitrosylated protein assay. Total protein nitrosothiol concentration was determined by first lysing cells with hypotonic buffer (20 mM β-glycerophosphate buffer pH 7.4 containing 50 mM NEM). Then lysates were centrifuged at 15,000 x g for 30 minutes and the soluble material was dialyzed for 5 days at 4°C against 20 mM β-glycerophosphate buffer pH 7.4. Dialyzed samples were analyzed for mercuric chloride-releasable nitrite by the methodology of Kostka and Park (31). Nitrite and S-nitrosoglutathione standards were prepared within 2 hours of the assay.

Total S-thiolated protein assay. Total S-thiolated protein was determined as described by Fariss and Reed (29). Briefly, treated cells were lysed with hypotonic buffer (20 mM β-glycerophosphate buffer pH 7.4 containing 50 mM NEM). Lysates were centrifuged at 15,000 x g for 30 minutes. Soluble proteins were then precipitated by adding 70% perchloric acid to a final concentration of 10%. After centrifugation, the supernatant was discarded and the pellet was washed with ice-cold 100% ethanol. The pellet was then resuspended in MOPS buffer + DTT or MOPS buffer alone and incubated for 60 minutes at 37°C. 70% perchloric acid was again added to a final concentration of 10% and the sample was centrifuged at 15,000 x g. The supernatant was recovered and released cysteine and GSH were analyzed by anion exchange HPLC as described above.

Isoelectric focusing (IEF) of phosphorylase b. Purified phosphorylase b was separated on horizontal slab gels (5.0% acrylamide/2.7%Bis-acrylamide/0.3% ampholyte pH 4.0-6.0/1.7% ampholyte pH 5.0-8.0) at 1500V and 1.1 watt/cm for 50 minutes (6). Approximately 1 μg of protein was applied to each lane.

Specific activity of cellular glutathione. Cells labeled with Tran^{35}S-label (ICN Pharmaceuticals, Inc., Costa Mesa, CA) were lysed and treated as above for analysis of low molecular weight thiols. Samples were prepared for anion exchange HPLC analysis of low molecular weight thiols as described. The GSH and GSSG peaks were collected and counted using a Beckman LS-100C scintillation counter.

Immunoprecipitation of H-Ras. H-Ras was immunoprecipitated from cellular lysates of NIH-3T3 cells overexpressing H-Ras using agarose-conjugated Ab-1. The agarose-conjugated Ab-1 was washed once with immunoprecipitation buffer (TBS/1% triton X-100/0.5% Sodium Deoxycholate/0.1% SDS) before adding to cellular lysates. The cells were washed twice with ice cold PBS and lysed with immunoprecipitation buffer containing 50 mM NEM. Lysates were centrifuged at 15,000 x g and the supernatant was incubated at 4°C with the agarose-conjugated Ab-1 for 2 hours. The agarose-conjugated Ab-1 was then washed twice in immunoprecipitation buffer and then layered over immunoprecipitation buffer + 7.5% sucrose twice. Approximately 5 µl of agarose-conjugated Ab-1 was used per 0.5 mg total protein to yield 0.1-0.2 µg of H-Ras.

Western Blot Analysis of H-Ras. H-Ras was separated by SDS-PAGE as previously described (32) and transferred to Immobilon-P (PVDF) (Millipore, Inc, Bedford, MA) membrane using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA) according to the recommendation of the manufacturer. H-Ras was visualized with Ab 146 primary antibody (1:3,000 dilution) and rat antimouse alkaline phosphatase secondary Ab (1:10,000). Bands were detected with p-nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indoyl phosphate (BCIP).

Assay of S-glutathiolated H-Ras. S-glutathiolation of H-Ras was determined using a modification of previously described methods (29). H-Ras was immunoprecipitated from cellular lysates as described above. The washes from the immunoprecipitation procedure were counted by liquid scintillation and one extra wash in immunoprecipitation buffer was added to bring unbound counts to background levels. H-Ras bound to agarose-conjugated Ab-1 was then precipitated by adding 70% perchloric acid to a final concentration of 10%. After centrifugation, the supernatant was discarded and the pellet was washed with ice-cold 100% ethanol. The pellet was then resuspended in MOPS buffer + DTT or MOPS buffer alone and incubated for 60 minutes at 37°C. 70% perchloric acid was again added to a final concentration of 10% and the sample was centrifuged at 15,000 x g. The supernatant was recovered and released radioactivity was counted by liquid scintillation. The radioactive counts of samples not treated with DTT were subtracted from the counts of samples treated with DTT to determine reduction sensitive counts. This number was divided by the specific activity of GSH to calculate the amount of S-glutathiolated protein. An aliquot of each experimental treatment was separated by SDS-PAGE as described (32) and transferred membrane for western blot analysis as described above The protein content of each assay was calculated from the SDS-PAGE/Western Blot by comparison with a dilution series on the same gel (not shown). When the membrane was exposed to film for 1 day or 5 days, the radioactivity associated with the protein allowed us to assess the purity of the immunoprecipitate as greater than 95% because no bands other than H-Ras were seen on the film.

Results:

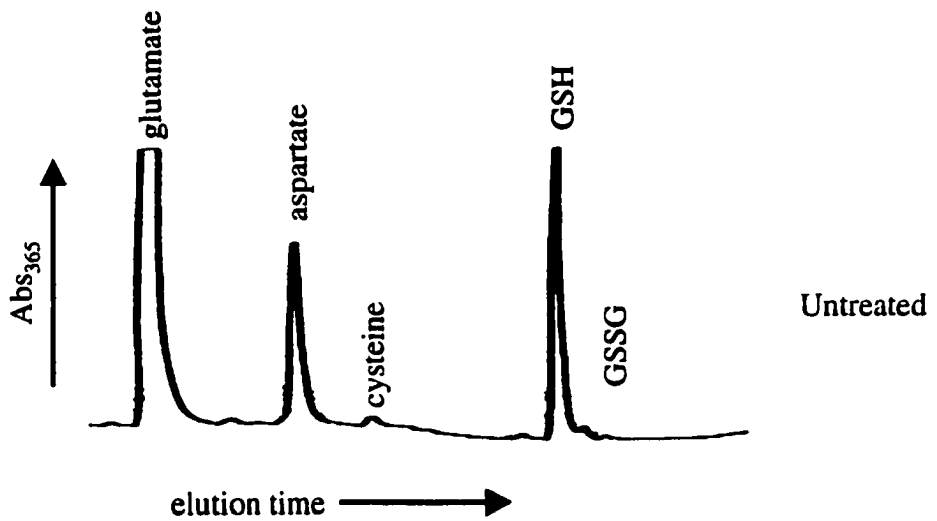
S-nitrosocysteine causes S-nitrosylation and S-thiolation of low molecular weight thiols in NIH-3T3 cells. In order to study S-thiolation and S-nitrosylation in cells, it was important to measure S-nitrosothiols and oxidized thiols simultaneously. Figure 1A shows the anion exchange HPLC analysis of the low molecular weight thiols of NIH-3T3 cells. GSH was the most abundant thiol present in these cell lysates. The concentration of cysteine, the next most abundant thiol, was approximately 10-fold lower than that of GSH. Several peaks appeared after adding 1mM S-nitrosocysteine for 3 minutes (Figure 1B). The new peaks were identified as described in the figure legend. Subsequent experiments in which the cellular thiols were labeled with ^{35}S-cysteine (Figure 1I) confirmed that all of the identified peaks were sulfur-containing compounds. The peaks for S-nitrosoglutathione, cystine and glutamate did not bind tightly to the column and were difficult to separate due to spreading of their peaks. Because of this, the sensitivity of the assay of S-nitrosoglutathione and cystine materials was diminished. S-nitrosocysteine could not be measured by this methodology. The S-nitrosoglutathione peak is evidence of S-nitrosylation of intracellular GSH. Increases in GSSG, cystine and cysteine-glutathione disulfide indicate that S-nitrosocysteine treatment led to formation of disulfides at the same time. Increases in intracellular total cysteine (cysteine + cystine + cysteine-glutathione disulfide) suggest that S-nitrosocysteine and not just the NO moiety entered the cell.

Figure 2A shows the time course of S-nitrosylation of GSH and the oxidation of the low molecular weight thiol pool by 1 mM S-nitrosocysteine. Intracellular S-nitrosoglutathione, GSSG, and cystine increased rapidly, reaching a maximum by 3 minutes and remained above background levels for greater than 60 minutes. GSH decreased ,

Figure 1. HPLC Analysis of low molecular weight thiol pool of NIH-3T3 cells with S-nitrosocysteine addition.

NIH-3T3 cells were treated with 1mM S-nitrosocysteine for 3 minutes, washed twice with ice cold PBS and lysed with 10% perchloric acid. The lysate was analyzed with anion exchange HPLC using the procedure of Fariss and Reed (29) modified for analysis of cystine, S-nitrosoglutathione, cysteine, cysteine-glutathione disulfide, GSH and GSSG as described in Materials and Methods. Compounds were identified by retention time, which was confirmed by HPLC analysis of the pure compounds. The peaks isolated from cells that coeluted with S-nitrosoglutathione, cystine, cysteine-glutathione disulfide and GSSG were reduced with DTT treatment. A. Lysate of untreated cells. B. Lysate of cells treated with 1mM S-nitrosocysteine for 3 minutes.

A.


glutamate
aspartate
GSH
Abs365
cysteine
GSSG
Untreated
elution time

B.

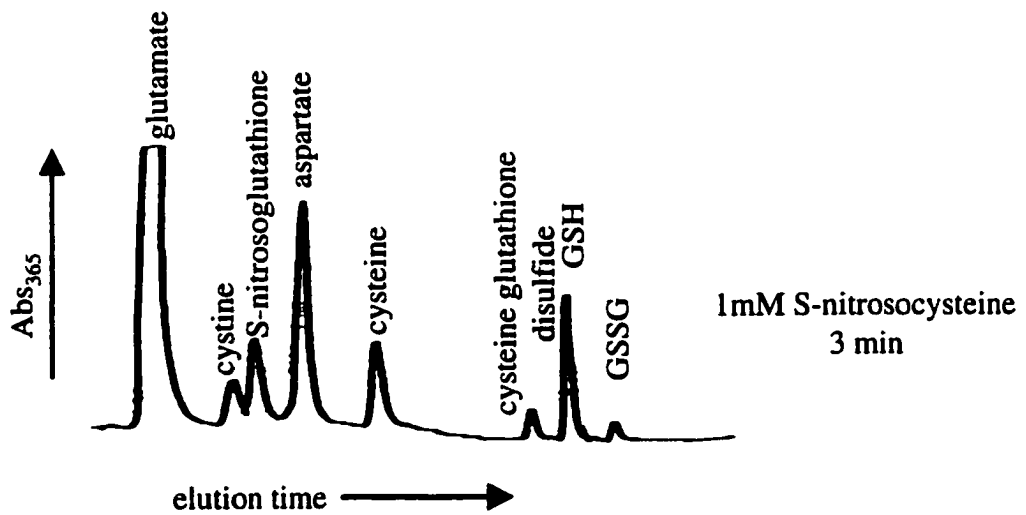
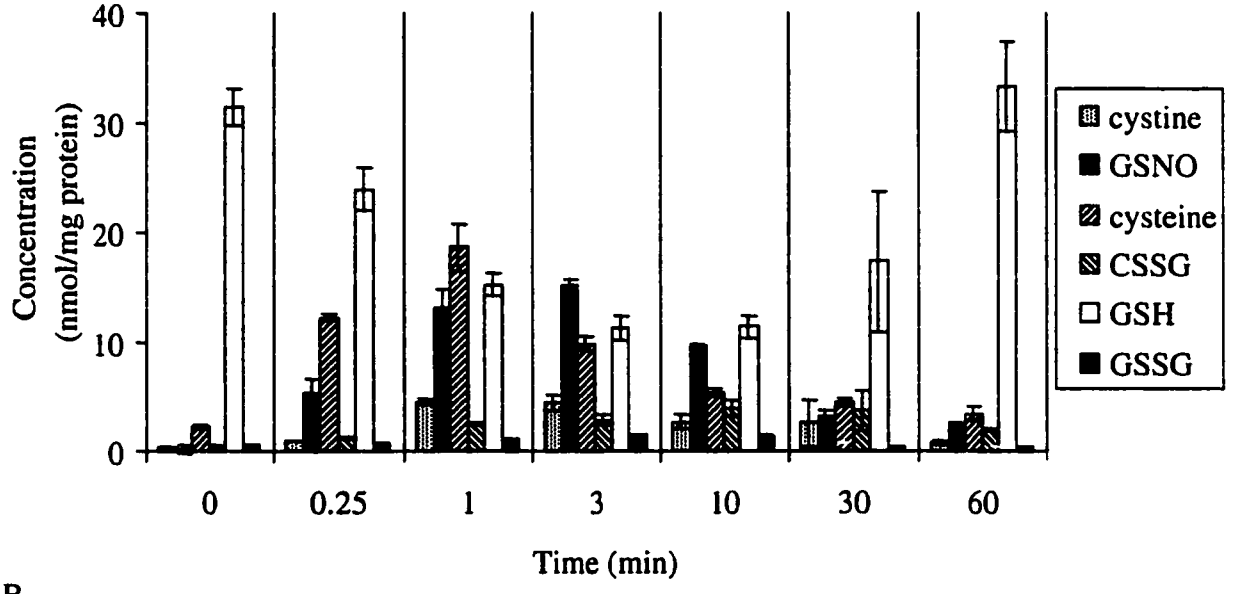

glutamate
cystine
S-nitrosoglutathione
aspartate
cysteine
cysteine glutathione disulfide
GSH
GSSG
Abs365
1mM S-nitrosocysteine
3 min
elution time

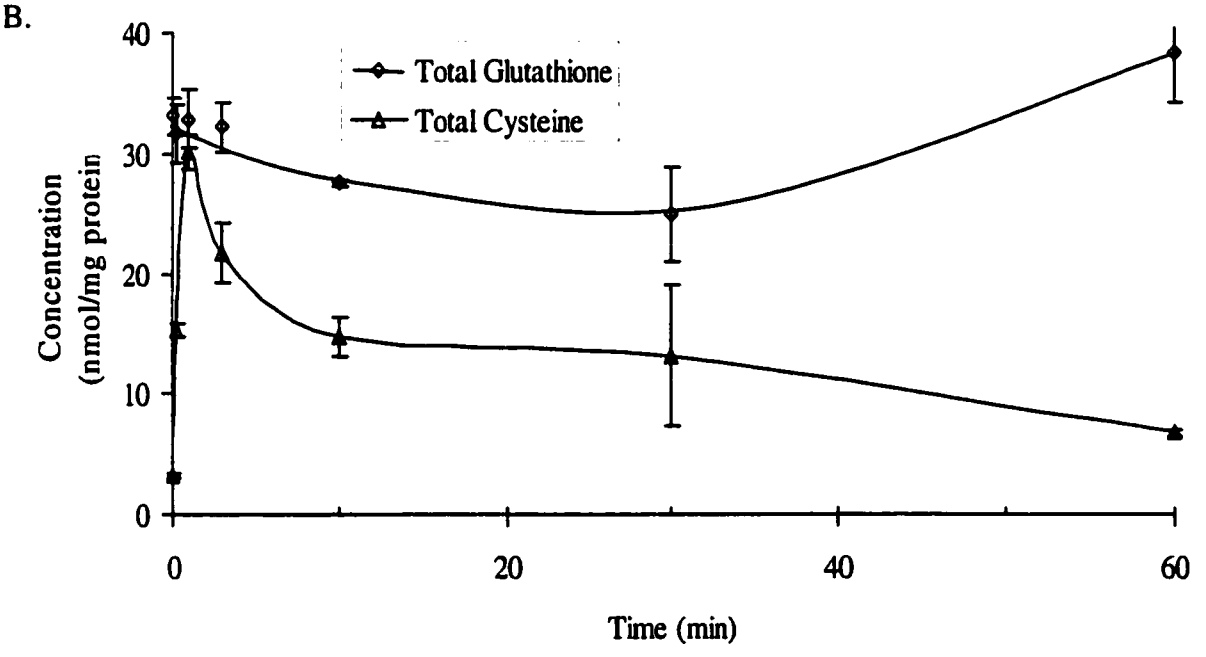
Figure 2. Time course of low molecular weight thiol changes in NIH-3T3 cells after S-nitrosocysteine addition.

A. Cells were incubated in 1 mM S-nitrosocysteine and lysed at the indicated times. Low molecular weight thiols were analyzed by anion exchange HPLC as described in Materials and Methods. Non-standard abbreviations: GSNO, S-nitrosoglutathione; CSSG, cysteine-glutathione disulfide. B. Time dependent changes in total glutathione and total cysteine from part A. Total glutathione = GSSG + cysteine-glutathione disulfide + S-nitrosoglutathione + GSH. Total cysteine = cystine + cysteine-glutathione disulfide + cysteine.

A.


Concentration (nmol/mg protein)
40
30
20
10
0
0
0.25
1
3
10
30
60
Time (min)
cystine
GSNO
cysteine
CSSG
GSH
GSSG

B.


Concentration (nmol/mg protein)
40
30
20
10
0
0
20
40
60
Time (min)
Total Glutathione
Total Cysteine

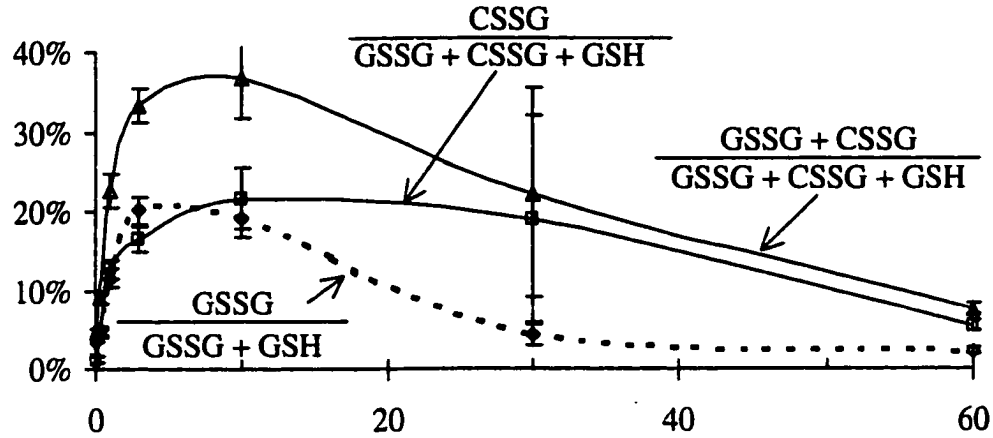
rapidlyreaching its lowest point by 3 minutes and returning to pretreatment levels by 60 minutes. Cysteine-glutathione disulfide increased more slowly until 10 minutes while cysteine reached its maximal levels very rapidly (1 minute). Total glutathione levels (including GSH, GSSG, cysteine-glutathione disulfide, and S-nitrosoglutathione) remained relatively constant after S-nitrosocysteine addition (Figure 2B). In contrast, total cysteine (cysteine, cystine, and cysteine-glutathione disulfide) increased by more than 10-fold within 1 minute of S-nitrosocysteine addition. Total cysteine remained well above normal levels for more than 60 minutes. This kinetic analysis demonstrates that S-nitrosocysteine action is very rapid in cells producing significant changes in a variety of important compounds.

Increased concentrations of oxidized forms of glutathione are indicative of intracellular oxidative stress. Figure 3 shows the rate of change in the oxidation of the total glutathione and cysteine pools in NIH-3T3 cells with 1 mM S-nitrosocysteine. Figure 3A summarizes effects on glutathione-containing molecules. In Figure 3A, the ratio of GSSG/(GSSG + GSH) is highlighted with a dotted line, since this ratio is frequently used to express the oxidation state of the total glutathione pool. A maximum of 20% of the total glutathione would be GSSG by this assessment. However, major amounts of the total glutathione are also present as cysteine-glutathione disulfide. If cysteine-glutathione disulfide is examined as a fraction of total glutathione (GSSG + cysteine-glutathione disulfide + GSH), it is clear that 20% of the total glutathione is still in the oxidized form even at 30 minutes after treatment. The physiological role of cysteine-glutathione disulfide has not been studied in cells, but it is obvious here that it is contributing significantly to the oxidation state of the glutathione pool. The time course of cysteine-glutathione disulfide formation indicates that it forms slightly later than the other oxidized compounds, that its

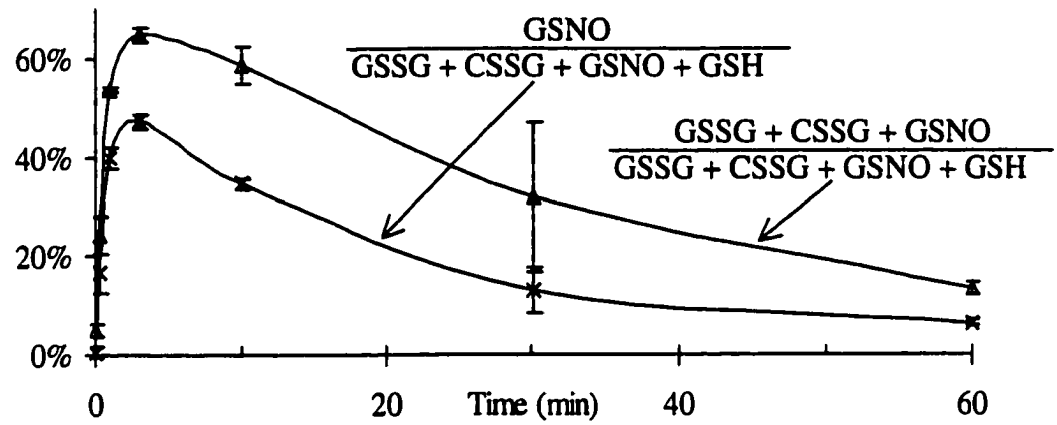
Figure 3. Time dependent changes in ratios of oxidized thiols with S-nitrosocysteine addition.

Fractions of oxidized thiols in NIH-3T3 cells caused by S-nitrosocysteine were calculated from data in Figure 2A. Non-standard abbreviations: GSNO, S-nitrosoglutathione; CSSG, cysteine-glutathione disulfide. A. Time dependent changes in disulfide formation of glutathione with S-nitrosocysteine addition. B. Time dependent changes in oxidation, including S-nitrosylation, of glutathione. C. Time dependent changes in disulfide formation of cysteine with S-nitrosocysteine addition.

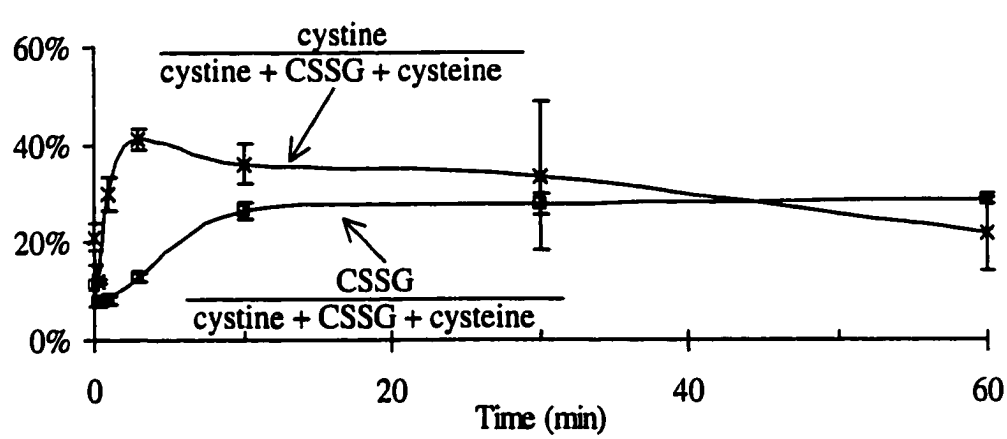
A.


CSSG
GSSG + CSSG + GSH
GSSG + CSSG
GSSG + CSSG + GSH
GSSG
GSSG + GSH
40%
30%
20%
10%
0%
0
20
40
60
Time (min)

B.


GSNO
GSSG + CSSG + GSNO + GSH
GSSG + CSSG + GSNO
GSSG + CSSG + GSNO + GSH
60%
40%
20%
0%
0
20
Time (min)
40
60

C.


cystine
cystine + CSSG + cysteine
CSSG
cystine + CSSG + cysteine
60%
40%
20%
0%
0
20
40
60
Time (min)

breakdown is less efficient than other compounds, or both. Cysteine-glutathione disulfide may be the product of the reduction of S-glutathiolated proteins by cysteine or of S-cysteylated proteins by glutathione. It may be broken down by participating in S-glutathiolation and S-cysteylation of protein sulfhydryls by thiol-disulfide exchange. Glutathione reductase may also reduce cysteine-glutathione disulfide, but it is possible that the kinetics of this reduction are different than the kinetics of reduction of GSSG by glutathione reductase. These factors may all contribute to the differences in time dependence between cysteine-glutathione disulfide and the other oxidized compounds.

In Figure 3B, the contribution of S-nitrosoglutathione to the total glutathione pool is analyzed. At the early times, S-nitrosoglutathione can account for about 50% of the total glutathione (GSSG + cysteine-glutathione disulfide + S-nitrosoglutathione + GSH) in the cells (Figure 3B). The other curve assesses the contribution of the total oxidized forms and it is clear that other oxidized thiols (i.e. disulfides) form the bulk of oxidized glutathione at 30 minutes and 60 minutes. Thus, S-NO bonds are more abundant early and disulfide bonds are more important late in the process.

The influx of cysteine with S-nitrosocysteine addition to cells suggests that the oxidation state of the cysteine pool might be important (Figure 3C). Cystine was the more important of the two oxidized forms at early times and cysteine-glutathione disulfide became a more significant fraction at 10, 30 and 60 minutes. Cysteine-glutathione disulfide therefore contributes significantly to the oxidation state of both the total glutathione and cysteine pools.

Table I shows the effect of the concentration of S-nitrosocysteine on changes in cellular thiol pools. The amount of total cysteine in the cells (cysteine + cystine + cysteine-glutathione

Table I

Effect of S-nitrosocysteine concentration on low molecular weight thiols in NIH-3T3 cells.

S-nitrosocysteine was added to NIH-3T3 cell cultures in three different concentrations and low molecular weight thiols were analyzed by anion exchange HPLC as described in Materials and Methods. Data are reported in units of nmol/mg total protein.

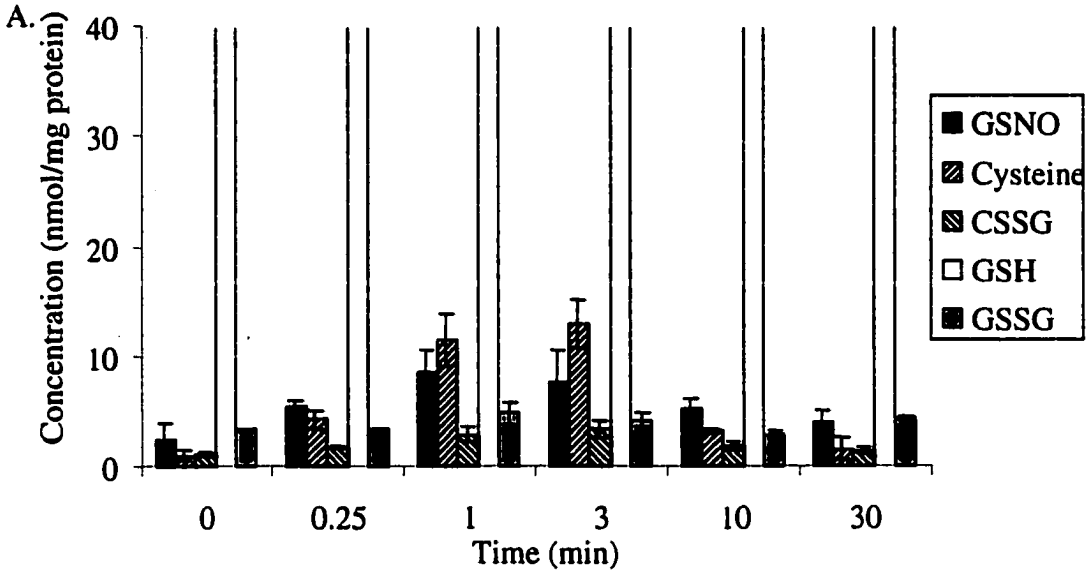
Conc. (mM)	time (min)	cysteine (nmol/mg)	cystine (nmol/mg)	CSSG (nmol/mg)	GSNO (nmol/mg)	GSH (nmol/mg)	GSSG (nmol/mg)
—	0	0.6±0.2	<0.1	0.4±0.3	<0.1	27±4	1.3±0.2
0.1	10	7.4±0.2	<0.1	0.9±0.1	0.3±0.6	27±2	0.75±0.15
	30	5.1±0.3	<0.1	0.7±0.1	<0.1	25±2	0.74±0.11
	60	3.6±0.2	0.5±0.9	0.6±0.1	0.2±0.4	30±2	0.87±0.13
1	10	8.9±0.7	4.9±0.2	5.6±0.1	5.7±0.4	7.7±0.3	0.94±0.08
	30	8.0±1.4	3.2±0.7	4.5±0.7	2.5±0.4	12±2	0.49±0.10
	60	4.0±0.3	1.2±0.3	2.5±0.2	2.2±0.1	21±2	0.79±0.08
10	10	0.6±0.5	75±4	13±1	<0.1	3±2	0.23±0.05
	30	0.9±0.2	113±9	13±1	<0.1	2±2	0.20±0.07
	60	0.6±0.3	140±10	12±3	<0.1	7±2	<0.1

disulfide) rose dramatically with concentration. At low S-nitrosocysteine, this pool was mostly reduced, while at high S-nitrosocysteine concentrations, it was completely oxidized to the disulfide. Cysteine-glutathione disulfide rose significantly after S-nitrosocysteine addition at all concentrations. S-nitrosoglutathione concentration was highest with 1 mM S-nitrosocysteine. At high S-nitrosocysteine concentration, the total glutathione pool was significantly depleted and what remained was primarily cysteine-glutathione disulfide. Because different cell types contain variable amounts of soluble thiols (33), the effect of S-nitrosocysteine on primary hepatocytes was studied. Hepatocytes contain approximately four times the amount of total glutathione that is found in NIH-3T3 cells. Figure 4A shows increases in intracellular S-nitrosoglutathione, cysteine, and cysteine-glutathione disulfide with S-nitrosocysteine addition. GSH concentration was approximately 120 nmol/mg protein and was essentially unchanged in this experiment. S-nitrosocysteine had a similar effect to that seen in NIH-3T3 cells, but the changes lasted for a shorter time. Changes in total glutathione (Figure 4B) were much smaller than those in NIH-3T3 cells. Total cysteine concentration rose significantly at early times but returned to pretreatment levels by 10 minutes. Figure 5A,B show that the total glutathione pool was mostly reduced in hepatocytes. Cysteine-glutathione disulfide (Figure 5A, bottom curve) was not as great a factor in determining the disulfide bonding state of the glutathione pool (Figure 5A, top curve) as it was in NIH-3T3 cells (Figure 3A.). It did have different kinetics of formation from GSSG (Figure 5A, middle curve) or S-nitrosoglutathione (Figure 5B, bottom curve). S-nitrosoglutathione accounted for slightly less than 50% of the total oxidized glutathione (Figure 5B top curve) and was the most abundant oxidized form of glutathione in

Figure 4. Effect of S-nitrosocysteine on low molecular weight thiols in rat primary hepatocytes.

A. Cultured primary hepatocytes were prepared according to established protocols (34) and treated with 1 mM S-nitrosocysteine for the indicated times. Low molecular weight thiols were analyzed in cellular lysates by anion exchange HPLC as described in Materials and Methods. Cystine could not be resolved in hepatocyte lysates. Non-standard abbreviations: GSNO, S-nitrosoglutathione; CSSG, cysteine-glutathione disulfide. B. Time dependent changes in total glutathione (GSSG + cysteine-glutathione disulfide + S-nitrosoglutathione + GSH) and total cysteine (cysteine-glutathione disulfide +cysteine) calculated from data obtained in part A of this figure.

A.


Concentration (nmol/mg protein)
40
30
20
10
0
0
0.25
1
3
10
30
Time (min)
GSNO
Cysteine
CSSG
GSH
GSSG

B.

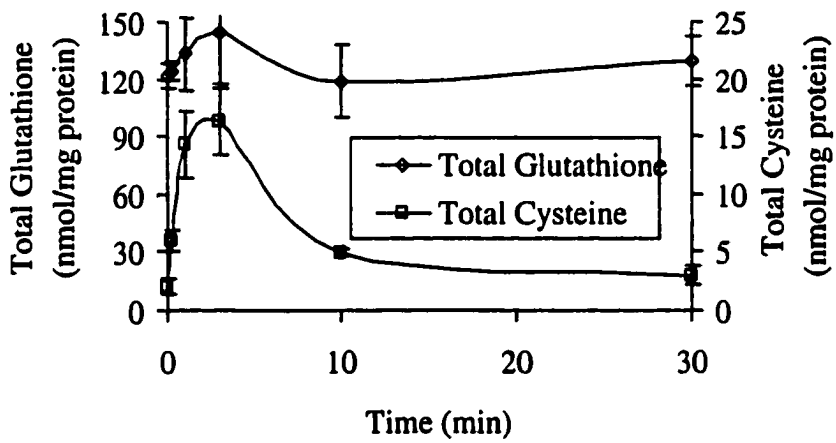
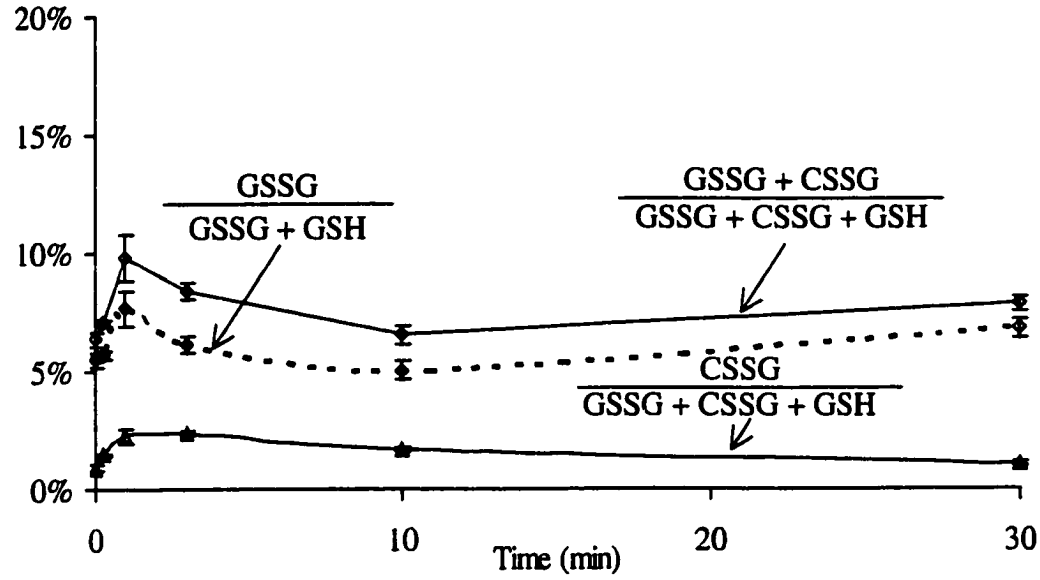

Total Glutathione (nmol/mg protein)
150
120
90
60
30
0
25
20
15
10
5
0
Total Cysteine (nmol/mg protein)
Total Glutathione
Total Cysteine
0
10
20
30
Time (min)

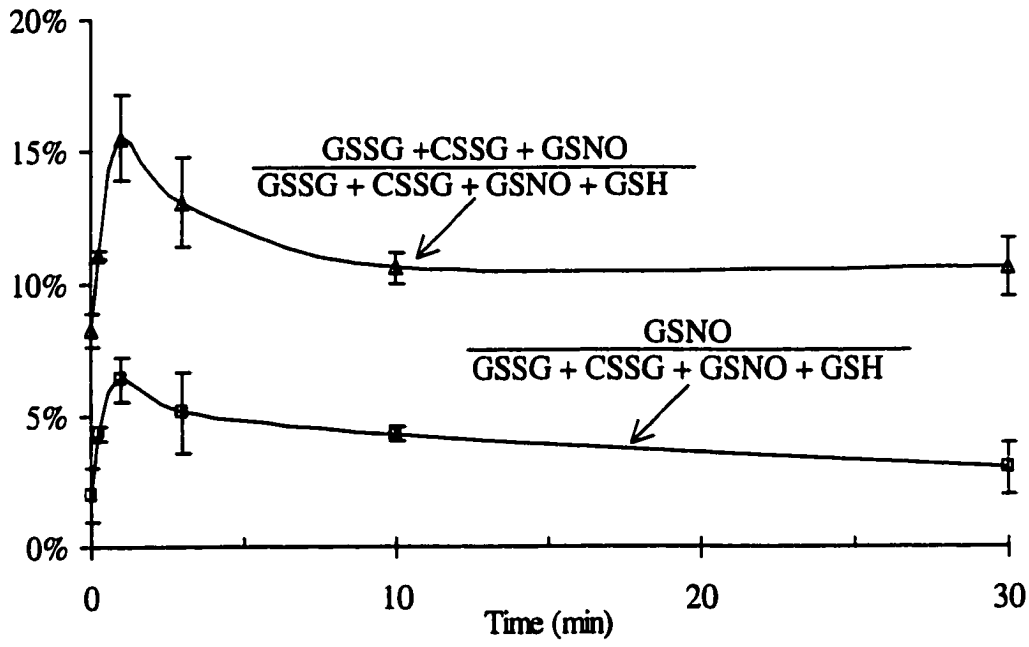
Figure 5. Time dependent changes in fraction of oxidized glutathione with S-nitrosocysteine addition in primary hepatocytes.

The fractions of oxidized glutathione in hepatocytes after S-nitrosocysteine addition were calculated from data in Figure 4A. Non-standard abbreviations: GSNO, S-nitrosoglutathione; CSSG, cysteine-glutathione disulfide. A. Time dependent changes in disulfide formation of glutathione with S-nitrosocysteine addition. B. Time dependent changes in oxidation, including S-nitrosylation, of glutathione.

A.


20%
15%
10%
5%
0%
GSSG
GSSG + GSH
GSSG + CSSG
GSSG + CSSG + GSH
CSSG
GSSG + CSSG + GSH
0
10
20
30
Time (min)

B.


20%
15%
10%
5%
0%
GSSG +CSSG + GSNO
GSSG + CSSG + GSNO + GSH
GSNO
GSSG + CSSG + GSNO + GSH
0
10
20
30
Time (min)

hepatocytes.

Overall, the low molecular weight thiol pool returned to normal before 30 minutes after addition of S-nitrosocysteine to hepatocyte cultures. This data suggests that effects of S-nitrosocysteine on cellular thiols can vary quantitatively with cell type, but the phenomena of S-nitrosylation and oxidation of intracellular thiols remains qualitatively the same. The much greater concentration of GSH in these cells probably played a large role in counteracting effects of S-nitrosocysteine.

Effect of S-nitrosoglutathione and S-nitroso-N-acetylpenicillamine on NIH-3T3 cells. S-nitrosoglutathione and S-nitroso-N-acetylpenicillamine are S-nitrosothiols that act as vasodilators *in vivo* (12,18). Like S-nitrosocysteine, they are commonly used to deliver NO in a variety of experiments (3,9,12). Intracellular effects of these two S-nitrosothiols on low molecular weight thiols were therefore assessed for comparison to the effects of S-nitrosocysteine.

Incubation of cells with 0.1 mM S-nitrosoglutathione produced no measurable changes in intracellular low molecular weight thiols (Table II). Increasing S-nitrosoglutathione to 1.0 mM did cause some increases in intracellular S-nitrosoglutathione, but did not change GSH (Table II), cystine, cysteine or cysteine-glutathione disulfide (not shown). Treatment with 10 mM S-nitrosoglutathione caused a dramatic increase in intracellular S-nitrosoglutathione and significantly depleted GSH. Only 10 mM S-nitrosoglutathione treatment caused a significant increase in the fraction of oxidized glutathione. Both S-nitrosoglutathione and GSSG levels were much greater than GSH levels after 30 minutes. This indicates that the cells were unable to effectively metabolize S-nitrosoglutathione at 10 mM despite only slight changes associated with 1 mM S-nitrosoglutathione treatment. Whether this is because of loss

Table II

Effect of S-nitrosoglutathione and S-nitroso-N-acetylpenicillamine on low molecular weight thiols in NIH-3T3 cells.

S-nitrosoglutathione (GSNO) or S-nitroso-N-acetylpenicillamine (SNAP) was added to NIH-3T3 cell cultures in the indicated concentrations and low molecular weight thiols were analyzed by anion exchange HPLC as described in Materials and Methods. Cysteine, cysteine glutathione disulfide and cystine concentrations were unchanged (not shown). Data are reported in units of nmol/mg total protein.

Treat.	Conc. (mM)	time (min)	GSNO (nmol/mg)	GSH (nmol/mg)	GSSG (nmol/mg)
GSNO	—	0	0.50±0.04	14±1	0.45±0.09
	0.1	10	0.4±0.1	11±2	0.32±0.03
		30	0.41±0.05	12±1	0.42±0.05
		60	0.5±0.2	15±0	0.35±0.06
	1	10	0.90±0.02	12±3	0.4±0.1
		30	1.4±0.1	15±0	0.49±0.03
		60	1.3±0.2	12±1	0.47±0.07
	10	10	11±4	8.7±0.1	1.5±0.3
		30	23±1	0.5±0.3	2.5±0.9
		60	40±30	0.1±0.2	5±3
SNAP	—	0	0.12±0.03	20±3	0.25±0.03
	1	10	0.24±0.09	21±5	0.30±0.09
		30	0.29±0.08	17±4	0.28±0.02
		60	0.5±0.2	14±1	0.22±0.03
	10	10	2.1±0.4	13±1	0.21±0.03
		30	2.8±0.5	8±2	0.19±0.02
		60	2.6±0.3	13±2	0.20±0.01

of cellular integrity or through some other mechanism was not explored. S-nitrosoglutathione caused increases in intracellular S-nitrosoglutathione much more slowly than did S-nitrosocysteine. This suggests that S-nitrosoglutathione does not enter the cell as readily as S-nitrosocysteine, and that significant amounts of S-nitrosoglutathione remained outside of the cells for at least 30 minutes.

S-nitroso-N-acetylpenicillamine (0.1 mM) caused no measurable changes in either GSH or S-nitrosoglutathione (not shown). Addition of 1mM S-nitroso-N-acetylpenicillamine caused a much smaller increase in intracellular S-nitrosoglutathione (Table II) than did treatment with 1 mM S-nitrosocysteine (Table I). Increasing S-nitroso-N-acetylpenicillamine concentration to 10 mM produced significant S-nitrosylation of GSH. Both 1 and 10 mM S-nitroso-N-acetylpenicillamine caused depletion of the GSH pool over time. While the fraction of glutathione in the form of GSSG did not change at any concentration, S-nitrosoglutathione accounted for a significant portion of the total glutathione at 10 mM S-nitroso-N-acetylpenicillamine (Table II). S-nitroso-N-acetylpenicillamine caused no measurable changes in cystine, cysteine or cysteine-glutathione disulfide at any concentration tested (not shown). The effects of S-nitroso-N-acetylpenicillamine in cells were nearly maximal after 10 minutes, but were sustained throughout the entire 60 minutes. Overall, equal concentrations of S-nitroso-N-acetylpenicillamine had much less effect on low molecular weight thiols than either S-nitrosocysteine or S-nitrosoglutathione.

If 1 mM cysteine was added to the cell cultures simultaneously with 1 mM S-nitrosoglutathione, intracellular S-nitrosoglutathione, cysteine, cystine and cysteine-glutathione disulfide increased rapidly (Figure 6). This effect was similar to 1 mM S-nitrosocysteine alone. L-cysteine or D-cysteine were both effective. This suggests that

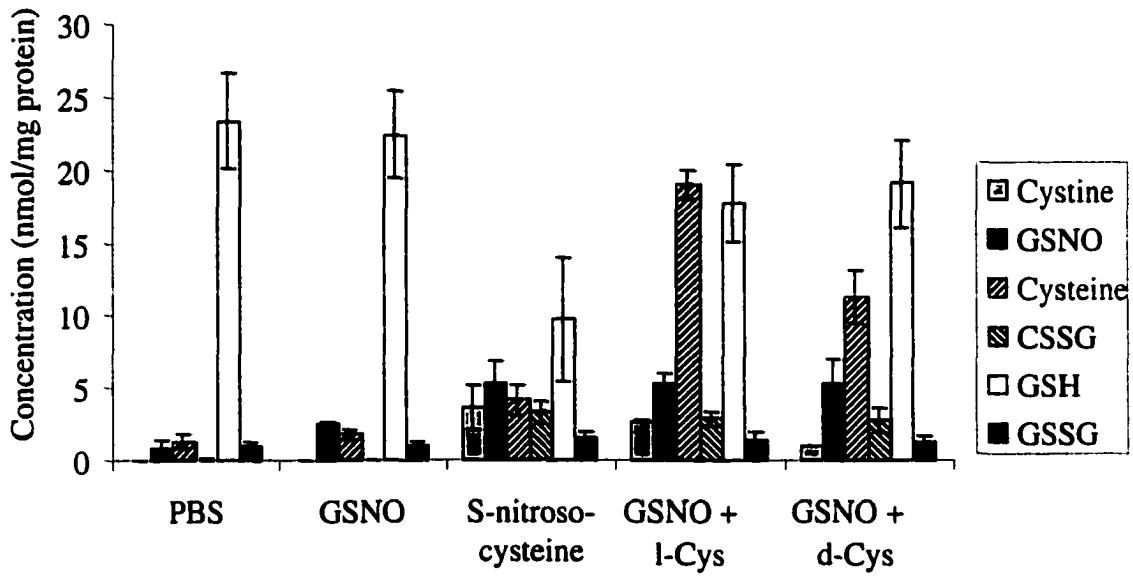


Figure 6. Comparison of S-nitrosoglutathione, S-nitrosocysteine or S-nitrosoglutathione/cysteine effects on low molecular weight thiols.

NIH-3T3 cells were treated with 1 mM S-nitrosoglutathione, 1 mM S-nitrosocysteine, 1 mM S-nitrosoglutathione + 1 mM L-cysteine, or 1 mM S-nitrosoglutathione + 1 mM D-cysteine for 10 minutes. Low molecular weight thiols were analyzed by anion exchange HPLC as described in Materials and Methods. Non-standard abbreviations: GSNO, S-nitrosoglutathione; CSSG, cysteine-glutathione disulfide.

S-nitrosoglutathione is S-nitrosylating cysteine, which then enters the cells and causes changes in the low molecular weight thiol pool. While intracellular S-nitrosoglutathione and cysteine-glutathione disulfide levels of S-nitrosocysteine-treated and S-nitrosoglutathione/cysteine-treated cells were similar, GSH was less depleted and cysteine increased more in the S-nitrosoglutathione/cysteine-treated cells. These differences may have been caused by free cysteine entering the cell, supplementing the intracellular cysteine pool and protecting the GSH pool from oxidation. Neither the increase in S-nitrosoglutathione nor the increases in cystine or cysteine-glutathione disulfide could be accounted for by reduced cysteine entering the cell.

S-nitrosylation and S-thiolation of soluble proteins in NIH-3T3 cells by S-nitrosocysteine. The biological effects of S-nitrosothiols may be caused by S-nitrosylation of protein thiols in cells (7,10,35). However, the effect of S-nitrosocysteine on low molecular weight thiols suggests both S-nitrosylation and S-thiolation of protein thiols might be important. S-thiolation and S-nitrosylation of protein thiols can be studied readily by methods already developed. Proteins are isolated by methods that block artifactual modification of cysteine (21). S-thiolation is measured by release of thiols by reduction (29), while S-nitrosylation is measured as mercuric chloride releasable nitrite (31). To test stability of S-nitrosylated proteins during dialysis, standards of reduced and S-nitrosylated phosphorylase b were prepared as previously described by Ji, et. al. (6). S-nitrosylation is assayed by inhibition iodoacetic acid alkylation as determined by isoelectric focusing (IEF) analysis (Figure 7). IEF separates proteins based on their net charge. For each alkylated cysteine on phosphorylase b, the protein migrates at a progressively more acidic pI. Phosphorylase b possesses four cysteine residues that react with iodoacetic acid (lane 1). IEF shows that most

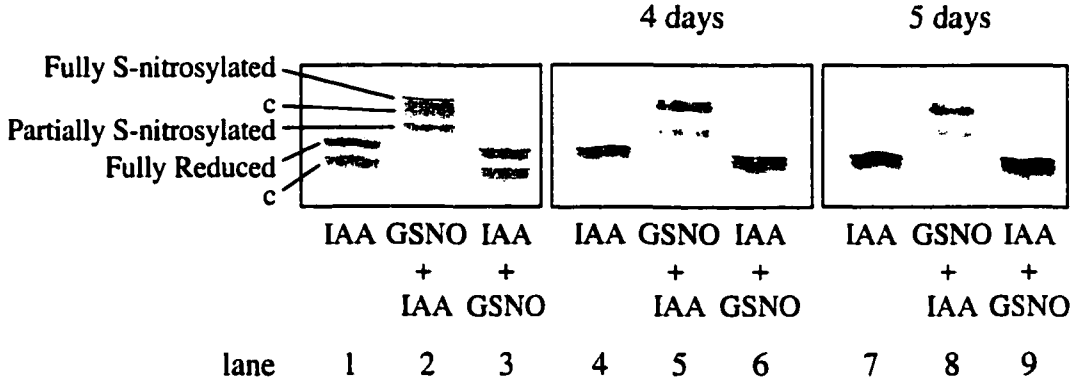


Figure 7. Stability of S-nitrosylated phosphorylase b with dialysis.

Phosphorylase b was incubated with iodoacetic acid (IAA) (lanes 1,4,7), S-nitrosoglutathione (S-nitrosoglutathione) followed by IAA (lanes2,5,8), or IAA followed by S-nitrosoglutathione (lanes3,6,9). S-nitrosoglutathione treatment was a 15 minute incubation at pH 7.4 and 37°C. Phosphorylase b was alkylated by a 20 minute incubation at 37°C with 40 mM IAA at pH 8.0. Samples were dialyzed to remove excess S-nitrosoglutathione for 4 or 5 days before the nitrite assay. Replicate samples were separated on IEF. S-nitrosylated and reduced phosphorylase b were identified as described (6). Bands marked "c" denote a contaminant protein in the preparation (36) that is removed upon dialysis.

of the protein is fully S-nitrosylated after S-nitrosoglutathione treatment, with some partially S-nitrosylated species evident (lane 2). If phosphorylase b is alkylated before S-nitrosoglutathione treatment, S-nitrosylation is prevented (lane 3). After 4 (lanes 4-6) or 5 (lanes 7-9) days dialysis, the distribution of bands between the fully S-nitrosylated and partially S-nitrosylated forms (compare lanes 2, 5, and 8) remains constant. The IEF data therefore shows that the protein was stable during dialysis. After 4 or 5 days of dialysis, 3.4 mols of S-nitrosothiol per mol phosphorylase b were found as determined by the nitrite assay. Analysis of soluble protein S-nitrosothiols from NIH-3T3 cell extracts showed similar recoveries from samples dialyzed 4 days compared to those dialyzed for 5 days (not shown).

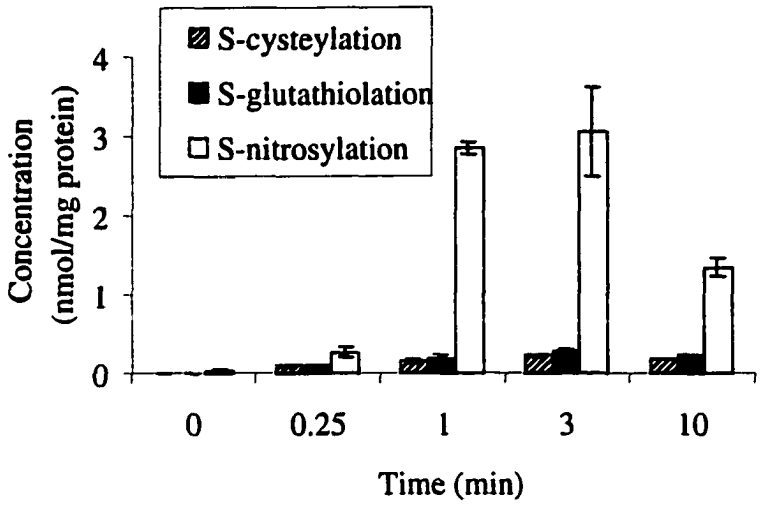
The kinetics of protein S-nitrosylation, S-glutathiolation, and S-cysteylation initiated by 1 mM S-nitrosocysteine were similar to those of the low molecular weight thiol pool (Figure 8A), peaking at 3 minutes and decreasing thereafter. S-nitrosylation was more than 10 times greater than S-glutathiolation or S-cysteylation. This decreased to about 5 times greater by 10 minutes. Figure 8B shows the concentration dependence of protein cysteine modification by S-nitrosocysteine. Slight increases of S-cysteylated and S-nitrosylated, but not S-glutathiolated proteins, occurred with 0.1 mM S-nitrosocysteine. Proteins were heavily S-nitrosylated after treatment by 10 mM S-nitrosocysteine and S-cysteylation became more prominent. While GSH was depleted after 10 mM S-nitrosocysteine treatments, protein sulfhydryls were not.

S-nitrosylation and S-glutathiolation of H-Ras in NIH-3T3 cells. Since S-nitrosocysteine was effective in causing S-nitrosylation, and, to a lesser extent, S-thiolation of protein thiols, we examined whether modification of H-Ras would also follow this pattern or whether specific proteins or specific cysteines would react differently. In order to study

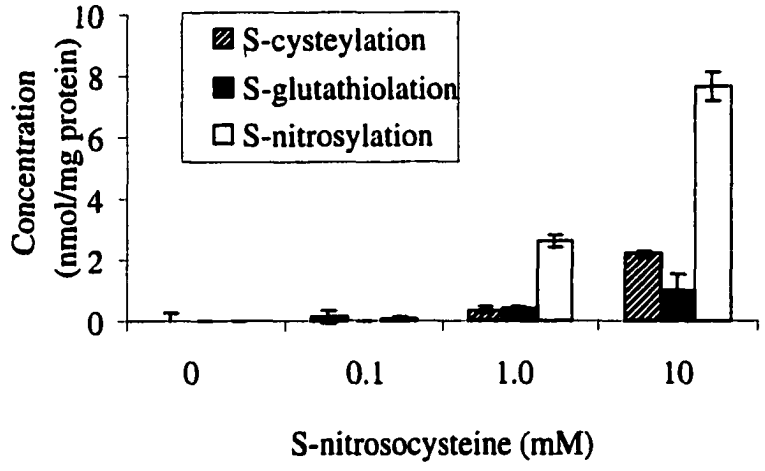
Figure 8. Total soluble protein S-cysteylation, S-glutathiolation, and S-nitrosylation of NIH-3T3 cells with S-nitrosocysteine addition.

A. Time course of protein cysteine modification with 1mM S-nitrosocysteine. Cells were incubated with 1 mM S-nitrosocysteine for the indicated times and lysed with 10 mM β-glycerophosphate buffer containing 50 mM N-ethylmaleimide. Total soluble protein S-glutathiolation or S-cysteylation was measured by trichloroacetic acid precipitation of soluble proteins followed by anion exchange HPLC of low molecular weight thiols released by DTT as described in Materials and Methods. Total protein S-nitrosylation was measured by nitrite assay of dialyzed cellular lysates as described in Materials and Methods. B. Concentration dependence of total soluble protein cysteine modification with 10 minute treatments with indicated concentrations of S-nitrosocysteine. Samples were processed as in part A.

A.


S-cysteylation
S-glutathiolation
S-nitrosylation
Concentration (nmol/mg protein)
0
1
2
3
4
0
0.25
1
3
10
Time (min)

B.


S-cysteylation
S-glutathiolation
S-nitrosylation
Concentration (nmol/mg protein)
0
2
4
6
8
10
0
0.1
1.0
10
S-nitrosocysteine (mM)

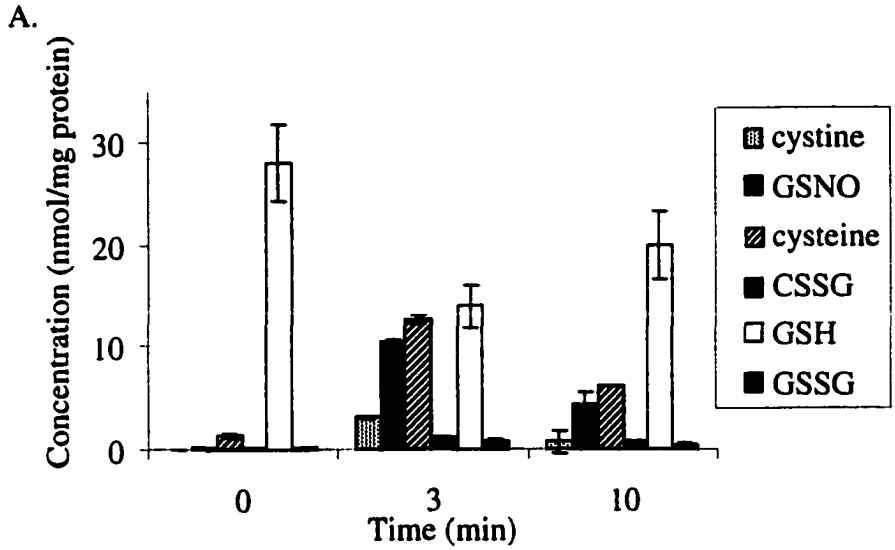
H-Ras *in vivo*, it was necessary to utilize cells that overexpressed H-Ras. Figure 9A shows effects of S-nitrosocysteine on low molecular weight thiols in NIH-3T3 cells overexpressing the wildtype H-Ras protein (NIH-3T3/WT). Since overexpression of H-Ras can have profound effects on the cellular morphology and metabolism (37), and possibly may also affect cellular response to S-nitrosocysteine treatment, it was important to establish the effect of S-nitrosocysteine on these cells. In spite of the fact that NIH-3T3/WT cells grew to a population that contained many more cells and approximately three times the total protein of the normal NIH-3T3 cells, the GSH concentration was similar to that of the parental NIH-3T3 cells. As in normal NIH-3T3 cells, S-nitrosocysteine caused rapid increases in S-nitrosoglutathione, cysteine, cystine and cysteine-glutathione disulfide and caused decreases in GSH. There are also rapid increases in S-nitrosylation, S-cysteylation, and S-glutathiolation of cytosolic proteins (Figure 9B). By these measures, overexpression of H-Ras does not significantly affect cellular responses to S-nitrosocysteine.

H-Ras was immunoprecipitated from these cells and the effect of S-nitrosocysteine was examined by methods described in the figure legend. Figure 10A shows that the immunoprecipitate contained primarily H-Ras. When the NO was determined on these immunoprecipitates, there was no evidence of S-nitrosylation of H-Ras in untreated cells, while in S-nitrosocysteine-treated cells, 43 nmols of S-nitrosothiol were detected per mg H-Ras (Figure 10B). This is equivalent to 0.85 moles S-nitrosothiol per mole of H-Ras in cells after S-nitrosocysteine treatment. H-Ras contained 43 nmol S-NO/mg of protein, as compared to 15 nmol S-NO/mg for the total cytosolic protein. Thus, H-Ras may be more

Figure 9. Thiol modification in NIH-3T3 cells overexpressing the H-Ras wildtype protein with S-nitrosocysteine addition.

A. Analysis of low molecular weight thiols upon addition of 1 mM S-nitrosocysteine for the indicated times. Thiols were quantified using anion exchange HPLC as described in Materials and Methods. Non-standard abbreviations: GSNO, S-nitrosoglutathione; CSSG, cysteine-glutathione disulfide. B. Total soluble protein S-cysteylation, S-glutathiolation and S-nitrosylation with addition of S-nitrosocysteine. Cells were treated with 1 mM S-nitrosocysteine for the indicated times and then lysed with 10mM β-glycerophosphate buffer containing 50 mM N-ethylmaleimide. Protein S-thiolation was measured by TCA precipitation of cytoplasmic proteins followed by anion exchange HPLC of DTT-releasable low molecular weight thiols as described in Materials and Methods. Protein S-nitrosylation was measured by nitrite assay of dialyzed soluble proteins as described in Materials and Methods.

A.


Concentration (nmol/mg protein)
30
20
10
0
0
3
10
Time (min)
cystine
GSNO
cysteine
CSSG
GSH
GSSG

B.

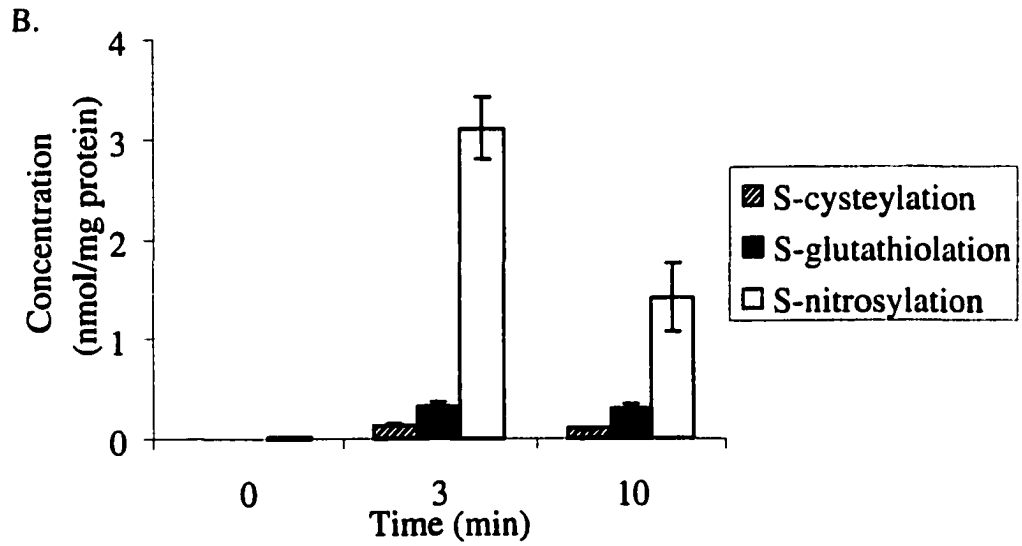
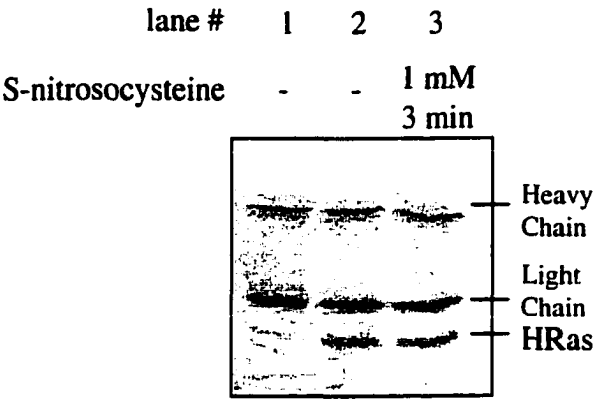

Concentration (nmol/mg protein)
4
3
2
1
0
0
3
10
Time (min)
S-cysteylation
S-glutathiolation
S-nitrosylation

Figure 10. S-Nitrosylation of H-Ras in NIH-3T3/WT cells with S-nitrosocysteine addition.

A. NIH-3T3 cells (lane 1) or NIH-3T3/WT cells (lanes 2 and 3) were incubated with PBS (lanes 1 and 2) or with 1 mM S-nitrosocysteine for 3 minutes (lane 3). Cells were washed twice with PBS and lysed with detergent-containing immunoprecipitation buffer + 50mM NEM. H-Ras protein was then immunoprecipitated from the extracts as described in Materials and Methods. One extra wash with PBS was added in order to remove detergents, which were found to interfere with the nitrite assay. The H-Ras bound to agarose beads was then analyzed for NO with the nitrite assay as described in Materials and Methods. Two samples of each immunoprecipitation were determined. An aliquot of each experimental treatment was separated by SDS-PAGE as described (32) and the protein content of each assay was calculated from the SDS-PAGE by comparison with a dilution series of purified H-Ras on the same gel (not shown). B. Analysis of S-nitrosylated H-Ras. H-Ras was immunoprecipitated from NIH/3T3 cells and S-nitrosylation of the immunoprecipitated protein was determined by nitrite release as described in part A of this figure. Duplicate samples of each treatment are shown. The protein content of each lane was determined as described in part A of this figure to normalize the NO content of each immunoprecipitate for the amount of H-Ras.

A.



B.

Lane	NO (pmol)	protein (μg)	NO/protein (pmol/μg)
1	15	n.d.	—
	4		—
2	9	1.25	7
	-9		-7
3	66	1.25	53
	40		32

sensitive to S-nitrosylation than the average cytosolic protein in cells.

To measure S-thiolation of H-Ras in these cells, the glutathione was labeled with ^{35}S-cysteine. After 24 hour labeling, the specific activity of GSH and cysteine was 6.5 x 10^7 cpm/μmol while GSSG and cysteine-glutathione disulfide were twice that (1.8 x 10^8 cpm/μmol) as is expected (Figure 11A). The S-glutathiolation of H-Ras was determined by assaying protein-bound radioactivity. Figure 11B shows that approximately 8 nmols of GSH were covalently bound to each mg of H-Ras upon S-nitrosocysteine treatment, or approximately 0.16 moles per mole of H-Ras. When compared to the 1.6 nmols/mg S-glutathiolation of total cytosolic proteins, this suggests that H-Ras is more readily S-glutathiolated than the average cytosolic protein.

The specific activity of GSH and GSSG was unchanged during the course of the experiment (Figure 11A), suggesting that the cysteine moiety of S-nitrosocysteine was not being used to synthesize GSH during this experiment. The specific activity of cysteine decreased 30-fold to 2.2 x 10^6 cpm/μmol. The specific activity of cysteine-glutathione disulfide became identical to that of GSH after 3 minutes because the radioactivity associated with cysteine was insignificant in comparison to that of glutathione. The specific activity of cysteine did not change after the initial drop at 3 minutes. Thus, S-nitrosocysteine was completely equilibrated with the cysteine pool before the 3 minute time point.

Discussion

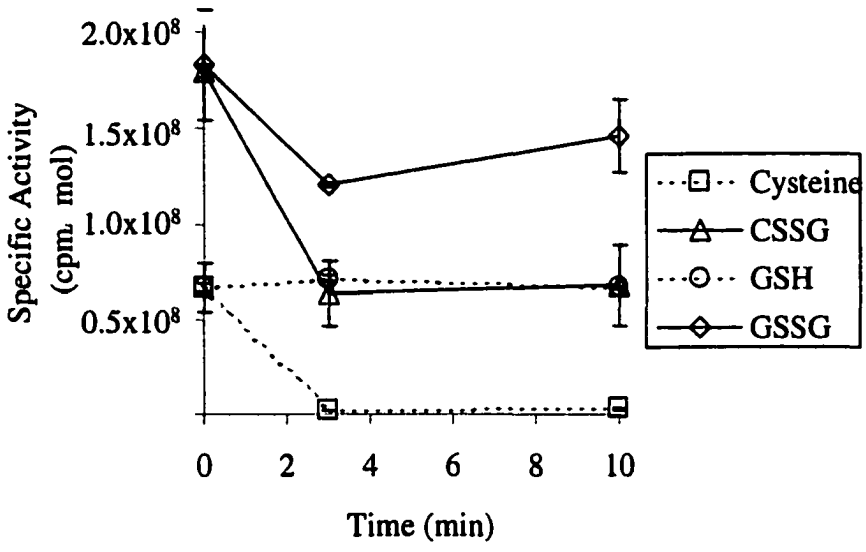
S-nitrosocysteine appears to be an S-nitrosothiol with unique properties. Our data support the idea that that the entire S-nitrosocysteine molecule enters the cell and reacts with

Figure 11. S-glutathiolation of H-Ras in NIH-3T3/WT cells with S-nitrosocysteine addition.

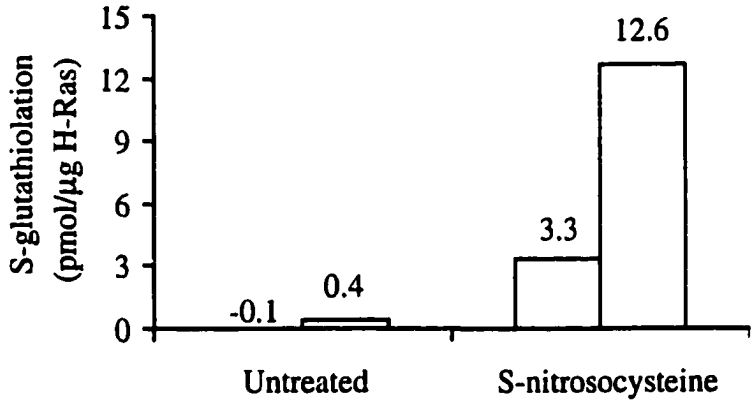
A. NIH-3T3 cells overexpressing WT H-Ras were incubated with Tran^{35}S-label for 24 hours and after 5 minute PBS incubation, were treated with 1mM S-nitrosocysteine. At the indicated times, cells were washed twice with ice cold PBS, lysed with 10% PCA and derivatized for low molecular weight thiol analysis as described in Materials and Methods. Specific activity was obtained as described in Materials and Methods. All data points were determined in duplicate. Non-standard abbreviation: CSSG, cysteine-glutathione disulfide.

B. NIH-3T3/WT cells were labeled with Tran^{35}S-label for 24 hours and subsequently treated with PBS or 1mM S-nitrosocysteine for 3 minutes. Cells were washed twice with ice cold PBS and then lysed with detergent containing immunoprecipitation buffer + 50mM NEM. H-Ras was then immunoprecipitated from the extracts and S-glutathiolated H-Ras was determined as described in Materials and Methods. The bar graph shows the data obtained from duplicate immunoprecipitations.

A.


2.0x10⁸
1.5x10⁸
1.0x10⁸
0.5x10⁸
Specific Activity
(cpm. mol)
0
2
4
6
8
10
Time (min)
Cysteine
CSSG
GSH
GSSG

B.


15
12
9
6
3
0
S-glutathiolation
(pmol/μg H-Ras)
-0.1
0.4
3.3
12.6
Untreated
S-nitrosocysteine

GSH and protein thiols. The decrease of specific activity of cysteine after addition of unlabeled S-nitrosocysteine to cells with an ^{35}S-labelled cysteine pool (Figure 11A) shows that cysteine from added S-nitrosocysteine is entering the cells. The specific activity of the cysteine pool does not change after 3 minutes, which means that the cysteine pool is equilibrated with the cysteine from S-nitrosocysteine by this time. The increase in S-nitrosoglutathione, cysteine, S-nitrosylated proteins, S-glutathiolated proteins, and S-cysteylated proteins, and the decrease in GSH all reached their maximum levels within 3 minutes. This suggests that the nitrosative and oxidative effects of S-nitrosocysteine addition to cells are dependent on the uptake of S-nitrosocysteine from the extracellular space. The increase in total cysteine as calculated by total decrease in specific activity of cysteine pool and the total increase in S-nitrosothiols (S-nitrosoglutathione + protein S-nitrosothiols) indicate that greater than 90% of total cysteine increase is accounted for by S-nitrosothiol increase. In other words, it appears that the assays presented here account for most of the S-nitrosocysteine entering the cell. Both nitrosylation and oxidation can therefore be considered primary events that result from uptake of S-nitrosothiols into cells.

Related compounds, S-nitrosoglutathione and S-nitroso-N-acetylpenicillamine are, on an equimolar basis, less effective oxidants and NO donors. Addition of cysteine to medium with S-nitrosoglutathione causes effects similar to addition of S-nitrosocysteine. Either D- or L-cysteine can mediate these effects. This suggests that S-nitrosocysteine is being taken into the cell at least partly via a non-stereospecific mechanism. Others have suggested that S-nitroso-D-cysteine may not taken up in cells as well as S-nitroso-L-cysteine (9,38). This may be a cell-specific phenomenon. Overall, data presented here agree with the hypothesis of a membrane transport role for S-nitrosocysteine postulated in previous reports (20).

While the S-nitrosylation of protein thiols was the dominant modification in response to nitrosative stress, a significant amount of protein S-thiolation was observed. The consequences of S-nitrosylation and S-thiolation on a protein might be similar if thiol modification inhibits the active site of a protein, as in the case of caspase (10), protein tyrosine phosphatase (39) or glyceraldehyde-3-phosphate dehydrogenase (GADPH) (19). However, it is more likely that allosteric effects, as suggested for H-Ras (7), would be mediated through addition of a large molecule carrying multiple charges such as glutathione, than a small, uncharged molecule like NO. Therefore, differentiating between these two modifications *in vivo* may be crucial to understanding the effects of S-nitrosothiol addition to cells.

Reports have been published about nitrosative and oxidative regulation of many diverse cellular functions (10,40,41). Among these are studies showing that the Erk pathway is upregulated in response to exposure of cells to diverse oxidative and nitrosative events (2-7). Several proteins along this pathway have been targeted for study with respect to this regulation (3,4,5,42). Among these proteins, the low molecular weight GTPase H-Ras was implicated by protein modification *in vitro* (43) and by mutational analysis *in vivo* (7). *In vitro* results in our lab suggested that H-Ras could be S-nitrosylated on as many as four sites (6,see Chapter II), while it could be S-glutathiolated on two sites (see Chapter II). Other labs have also shown that H-Ras is S-nitrosylated on at least one site *in vitro* (43). Within cells after treatment with S-nitrosocysteine, H-Ras is S-nitrosylated and S-glutathiolated to an equal or greater extent than the cytosolic protein pool (Figures 10 and 11). H-Ras is therefore a candidate for modulation of Erk pathway activity under oxidative/nitrosative conditions. It remains to be determined which sites are modified and what the effects of

modification are *in vivo*. Data in other labs indicates that S-nitrosylation of Cys118 of H-Ras would activate the Erk pathway. However, the fact that multiple cysteine residues, including the c-terminal cysteine residues that are normally lipidated, can be readily S-nitrosylated *in vitro* (6, see Chapter II) and S-thiolated *in vitro* and *in vivo* (see Chapter II) suggests that modulation of this pathway by H-Ras would be heavily dependent upon the source and dose of the oxidant stimulus in question.

In conclusion, this supports the idea that nitrosative modification of H-Ras occurs *in vivo*. It is the first report of S-glutathiolation *in vivo* of a specific protein in response to what was previously thought to be a nitrosative stimulus. This represents a first step in elucidating the primary events associated with initiation of redox regulation of signal transduction events *in vivo*.

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GENERAL SUMMARY AND CONCLUSIONS

Mechanism of antioxidant and regulatory properties of GSH

This dissertation provides evidence that GSH protects carbonic anhydrase III from irreversible damage by H_2O_2 or peroxyradicals. This extends the understanding of GSH-mediated protection of protein cysteines. It defines the concentrations at which GSH acts as an S-glutathiolating agent in concert with protein cysteines as opposed to a scavenger that does not interact with proteins. The concentration of GSH in cells is likely to be close to equimolar to the concentration of protein sulfhydryls. The data in chapter I show that S-glutathiolation is likely to occur at these GSH to protein ratios. This suggests that S-glutathiolation is at least as important for protection of proteins from damage, and is likely to be more important, than is direct scavenging of reactive oxygen species by GSH. The greater reactivity of H_2O_2 with H-Ras, in comparison to GSH (chapter II), while not conclusive in and of itself, agrees with this assessment.

GSH participates in oxidative modifications of protein cysteines at molar ratios of GSH:protein cysteine that are physiologically relevant. The role of GSH in protein oxidation is not merely that of a scavenger of oxyradicals as is widely believed, but as a co-participant in a fast, two step reaction between proteins, GSH and an oxidant. This means that in order for S-glutathiolation to occur, it is not necessary for GSSG to form. Thus, protein thiols may be oxidized before GSH is oxidized to GSSG. In this case, GSH converts the oxidized reactive protein thiol to the mixed disulfide. This prevents further oxidation of the protein thiol to irreversibly oxidized forms of cysteine and may have other implications.

S-glutathiolation and oxidation of H-Ras

H-Ras is believed to participate in relaying oxidative signals through the Erk pathway (52-57). Because the oxidants which had been shown to activate this pathway, H_2O_2, superoxide, the peroxynitrite donor, 3-morpholinosydnonimine (SIN-1), NO, S-nitroso-N-acetylpenicillamine, and sodium nitroprusside, all react readily with cysteine, cysteine oxidation might mediate this effect. One group of workers showed the modification of a truncated mutant of H-Ras with NO *in vitro* (56). This data, along with the activation of Erk in Jurkat T cells by NO led them to suggest that H-Ras was allosterically activated by S-nitrosylation of Cys118 *in vivo*. While this explanation was plausible, there remained no evidence of modification of H-Ras *in vivo* under oxidative or nitrosative conditions. Chapters II and III of this dissertation show that H-Ras, a critical protein component of the Erk signal transduction cascades, is modified both oxidatively and nitrosatively both *in vitro* and *in vivo*. This modification takes place on multiple thiols *in vitro* and the same is likely *in vivo*. This work presents evidence that H-Ras is readily modified on multiple thiols by several thiol oxidants, H_2O_2, S-nitrosoglutathione, GSSG, cystamine, and diamide *in vitro*.

We also show that H-Ras is S-thiolated in cells by the thiol oxidant diamide. This oxidation takes place on at least one of the cysteines which is normally lipidated, as indicated by the data that H-Ras lacking Cys118 is still S-thiolated. While oxidation of Cys118 may occur and activate H-Ras, oxidation of cysteines 181, 184 or 186 may also occur and inactivate H-Ras (48,49,56). This provides a plausible mechanism for both activation and inactivation of H-Ras by a single oxidant. This mechanism is of course dependent upon concentration of oxidant and the reactivity of individual cysteine residues. *In vivo*, this

modification would also be subject to the availability of cysteine residues, which may be limited by lipid modification.

Chapter III shows that H-Ras is S-nitrosylated in cells treated with S-nitrosocysteine. In agreement with *in vitro* data showing that H-Ras is somewhat more reactive than GSH towards H_2O_2 (Chapter II), H-Ras appears to be S-nitrosylated to a similar or greater extent than the average cytosolic protein (Chapter III). H-Ras is also S-glutathiolated in cells treated with S-nitrosocysteine. In this case, H-Ras seems to be significantly more S-glutathiolated than the average cytosolic protein (Chapter III).

Cell-type specific changes in response to oxidation

It is necessary to know the oxidation state of thiols in the cell to better predict mechanisms for activation or inactivation of a particular protein or pathway by an oxidant. The differences between response of a pathway, as is observed between different cell lines in Erk activation with oxidative and nitrosative stress (54), may be a result of the ability of a particular cell line to deal with a given stress. This is illustrated most simply in Chapter III in the difference in response of the primary hepatocytes to S-nitrosocysteine treatment when compared to the response of NIH-3T3 cells. The response to different concentrations of diamide was also altered depending on which H-Ras mutant was overexpressed in the cells (Chapter II). While the overexpression of H-Ras in NIH-3T3 cells did not seem to significantly affect the oxidative treatment with 1 mM S-nitrosocysteine at early time points (Chapter III), the response may have varied at different concentrations, and the recovery of the cell at later time points may have been altered.

Oxidative modulation of Erk pathway activity

The Erk pathway may be activated by modification of H-Ras but it may also be inactivated (48,49,53). While this study shows that by all criteria examined that H-Ras is readily modified in oxidative conditions, there are several other members of the Erk pathway that may be regulated by cysteine oxidation. For example, protein tyrosine phosphatases have been shown to be inhibited by S-glutathiolation and formation of protein cysteine sulfenic acids (14,34,35). Oxidation a tyrosine phosphatase may directly modify the phosphorylation state of the Erk proteins. The Raf proteins also contain cysteine-rich regions that are required for activity. Disruption of these regions by oxidation may be a mechanism for inactivation of the Erk pathway. Thus, a more quantitative assessment of oxidative effects on all of the proteins in the Erk pathway is necessary before one can determine the importance of modification of any one protein.

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